WATERLOGGING AND SALINITY TOLERANCE IN LUCERNE (*MEDICAGO SATIVA*)

by

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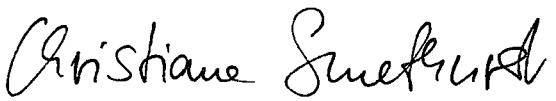
DEDICATION

This thesis is dedicated to my dear husband, Philip James Smethurst, and our three children, Benedict, Roland and Ingrid, who all encouraged me during trying times and supported me in this adventure.

Medicago sativa

DECLARATION

This thesis contains no material, which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge, contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.



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ABSTRACT

Lucerne (*Medicago sativa*) is a perennial crop with a deep taproot system penetrating several metres down the soil profile and thus can be integrated into crop rotations to reduce watertable recharge to ameliorate saline and waterlogged sites and to redress the environmental threat of rising saline watertables. Despite this fact, not much is known about specific mechanisms mediating adaptive responses of lucerne to waterlogging and salinity stresses. One of the aims of this project was to provide such information. Also, for lucerne's ameliorative properties to be fully exploited, tolerant genotypes need to be identified. Keeping in mind a high degree of genotypic variation within lucerne in terms of its tolerance to waterlogging and salinity, another aim of this study was to identify physiological indicators, which might become useful screening tools for tolerance to the stresses of waterlogging and salinity. Physiological responses to these abiotic stresses were characterized under a variety of conditions using photosystem (PS) II photochemistry, leaf gas exchange, pigment concentrations, biomass, nutrient dynamics and mesophyll cell anatomy, to find the most appropriate method for screening for stress tolerance in lucerne. The practical application of this work is its use as suggested screening tools for the ACIAR-funded project *Lucerne for Animal Production and the Environment*, (collaboration between Australia and China).

Significant effects of waterlogging were measured across all genotypes. Chlorophyll fluorescence variation became apparent on day 7 of stress and therefore appeared to be a useful, early non-destructive indicator of waterlogging stress tolerance. Recovery dynamics following waterlogging were investigated in four genotypes by studying PS II photochemistry and relating these to nutrient concentration changes. Nutrient concentrations at the end of recovery regained pre-stress values and PSII photochemistry also largely recovered. Preliminary studies on the concurrent stresses of waterlogging and salinity measuring chlorophyll fluorescence on excised, waterlogged leaves exposed to varying concentrations of salinity indicated a significantly greater stress response than salinity on its own.

Germination trials on ten genotypes and varying salinity levels pointed to a genotypic response to salinity, but correlations with parameters measured at later stages of plant ontogeny were not evident.

Salinity effects on plant growth characteristics, pigment and nutrient composition, leaf sap osmolality, changes in anatomical and electrophysiological characteristics of leaf mesophyll, and net ion fluxes in roots of six lucerne genotypes were investigated.

Waterlogging caused a marked reduction in photosynthetic capacity as measured with CO_2 assimilation rate and chlorophyll fluorescence. A wide range of Fv/Fm values (maximal photosynthetic capacity) was recorded within stressed cultivars, suggesting some scope in discovering more tolerant individuals. However, only minor genotypic differences were detected. This might be due to lucerne being a highly cross-pollinating species, as individual lucerne populations consist largely of a heterogeneous mixture of genetically heterozygous individuals. This fact seems to overshadow most of the differences between cultivars.

Results of the salinity experiment suggest that different lucerne genotypes use contrasting strategies to avoid toxic effects of sodium on cell metabolism. Sodium exclusion seemed to be used by at least one of the cultivars under investigation, whereas sodium inclusion and subsequent sequestering into vacuoles appeared to be used by other tolerant genotypes. When selecting genotypes for salt tolerance, it is important to consider the possibility of these different adaptation mechanisms being at play.

Overall, this study suggests that multiple traits are involved in determining salt and waterlogging tolerance in lucerne. The problem is additionally exacerbated by a high degree of genetic variability within heterozygous lucerne populations; therefore screening for lucerne improvement should rely on several, not just not one selection criteria. Ideally these criteria should be attributable to several physiological mechanisms involved. In addition, it appears that such screening should also be aimed at searching for outstanding individuals within a population, not only at comparing genotypes from a genetically diverse and promising pool of potentially tolerant lucerne germplasm.

PUBLICATIONS FROM THIS THESIS

Journal Papers

Smethurst C F and Shabala S 2003, Screening lucerne for waterlogging tolerance: comparative analysis of waterlogging effects on chlorophyll fluorescence, photosynthesis, biomass and chlorophyll content. **Functional Plant Biology 30,** 335-343

Smethurst C F, Garnett T and Shabala S 2004, Nutritional and chlorophyll fluorescence responses of lucerne (*Medicago sativa*) to waterlogging and subsequent recovery. **Plant and Soil** (in press)

Smethurst C F, Shabala S and Garnett T, Multiple traits associated with salt tolerance in lucerne: revealing the underlying cellular mechanisms. **Annals of Botany** (under submission)

Gill W M and Smethurst C F Effects of sodium chloride on ultrastructural changes in leaf mesophyll cells of lucerne (*Medicago sativa*) and concurrent nutrient changes in leaf tissue. **Biologia Plantarum** (in preparation)

Conference Presentations

Smethurst C F, Garnett T, Auricht G and Shabala S, The effect of waterlogging on chlorophyll fluorescence parameters in lucerne (*Medicago sativa*), 38th North American Alfalfa Improvement Conference in Sacramento, California in July 2002

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We have a beautiful
Mother
Her green lap
Immense
Her brown embrace
Eternal
Her blue body
Everything
We know
Alice Walker

Table of Contents

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

1. 1. INTRODUCTION

Agricultural production around the world is severely limited by waterlogging and salinity. Mild to moderate stresses may cause significant reduction in crop growth and yield, while more severe stresses result in plant death (Barrett-Lennard 2003). The economic cost of these losses is enormous. It is estimated that up to 20% of the global land area is affected by soil oxygen deprivation due to excessive water (Setter and Waters 2003) and thus result in reduced productivity. Direct yield losses of 2.5 t/ha have been measured in many crops (McFarlane and Wheaton 1990). Loss in total crop production due to waterlogging in an average rainfall year has been estimated at $A25 million per year in Western Australia alone (ACIAR for CIM/1996/025). The cost of drainage related soil degradation to Australian agriculture is estimated at more than $270 million each year and the cost to growers can be as much as $138/ha in some years (CSIRO online 2004). In south Asia waterlogging affects about 4.6 M ha, largely in the irrigated areas of India and Pakistan (FAO online 2004).

Two major types of waterlogging - periodic or constant - are usually distinguished. Each of these may be caused by several confounding factors. One of them is removal of perennial vegetation. Conversion of indigenous ecosystems to make way for agricultural production, has been implemented widely for example in the south west of Western Australia (Williamson *et al.* 1997), with an annual clearing rate of about 400 000 ha of land over an extended period. Seasonal excessive rainfall, snowmelts, river overflows and excessive irrigation, exacerbated by compaction and poor drainage are also potential causes of waterlogging stresses. Low-lying coastal regions are likely candidates for periodic flooding when large and even smaller rivers overflow their banks. Topographical

characteristics such as plains will slow the rate of lateral flow of surface water. Snowmelts as well as excessive irrigation can also lead to temporary hypoxia, especially when drainage is impeded due to relatively impermeable soils. Even well aerated soils have atmospheres with low concentrations oxygen, but when flooded they can be rapidly further depleted due to the respiration of roots and aerobic microorganisms (Larcher 1995).

Waterlogging and consequent hypoxia in agricultural regions is a growing problem, as crop plants (with the exception of rice) are poorly adapted to anaerobic conditions. Throughout the world there is increasing pressure on agricultural land to produce food crops for a growing population (Barrett-Lennard 2003; Sharma and Goyal 2003). This pressure can lead to degeneration of the land, because clearing of native, deep-rooted perennial vegetation, whose communal transpiration rate is much higher than that of agricultural crops that replace it, leads to a slow but steady rise of water tables. Under the new regime of agricultural cropping, precipitation is only partially used by the annual, shallow-rooted crops that transpire only for part of the year. During the rest of the year, water accumulates with the result of raising water tables from the subsoil towards the surface causing waterlogged, de-oxygenated conditions in surface soil.

High concentrations of salt in soils also account for a large decrease in crop yield both in Australia and world wide (Cocks 2003; Flowers and Yeo 1997; Greiner 1997). In 1998, the Prime Minister's Science, Engineering and Innovation Council estimated that the costs of dryland salinity include $700 million in lost land and $130 million annually in lost production (Walker *et al.* 1999). In Australia, about 30,000,000 ha of the mainland is underlain by salt (predominantly arid and semi-arid lands). Also, at least 4,000,000 ha are suffering from human-induced (secondary) salinisation. Of these, 156,000 ha are from irrigated land that is now totally unproductive, and 650,000 ha are in danger becoming so. In general, secondary salinisation in Australia is spreading at a compounding rate of about 5% per year (Haw *et al.* 2000). By 2020, somewhere between 10% and 25% of previously arable land could be out of production. In Western Australia it is estimated that 10% of the agricultural land is affected by salinity and it is expected that this will rise to 30% after several more decades

(McFarlane and Williamson 2002), mainly in the Southwest of the state. The salinity problem is a global one, however. For example, in south Asia alone, 42 M ha are affected by salinisation (FAO online 2004). Overall, approximately 6% of the world's land surface (Flowers and Yeo 1995) and 33% of irrigated land worldwide is affected by salinity (Ashraf 1994).

Several major types of salinity stresses may be distinguished. Primary salinity is defined as occurring naturally such as brackish biotopes of coastal regions or mangrove swamps and salt marshes. Primary salinity results from the accumulation of salts over long periods of time, through natural processes, such as weathering of parent material and also through accumulation of oceanic salt carried by wind and rain inland from coastal regions.

With increasing pressure on marginal land for food production saline coastal regions are being considered for crop production with resulting production restraints. Secondary salinity is caused by human-induced changes in the landscape. For example, as previously mentioned, deep-rooted perennials can reduce the recharge of groundwater, but shallow-rooted annual crops that transpire only part of the year allow water to "leak" to the watertable at a rate of about 150 mm annually (Cocks 2003). A rising watertable brings with it salt that was previously stored lower in the profile and was previously relatively immobile. Salt brought to the surface with the capillary rise of water is left on the exposed soil surface as the water is evaporated. These salts accumulate near the soil surface resulting in increasing salinity and eventual loss of arable land. Rising water tables are a feature of dryland salinity, which is spreading annually and rendering vast areas unproductive. Another cause of secondary-induced salinity is by irrigation schemes that either use poor quality (salty) irrigation water or where insufficient drainage causes raising of the watertable and subsequent salt mobilization. Worldwide vast areas of arable land are lost every year due to salinisation, particularly in arid regions. However, as increased salinisation of arable land is predicted to cause up to a 50% loss of farmland by 2050 (Wang *et al.* 2003) it is a priority to breed for salinity tolerance in crops.

Very often, salinity and waterlogging stresses occur together, not only in parts of the Australian landscape, but also in other areas of the world where secondary salinisation is a by-product of rising water tables. Also, the dual stress of waterlogging and salinity is a common feature in areas where native vegetation has been cleared. Although soil characteristics of waterlogged and saline sites may vary spatially and temporally, they lead ultimately to detrimental effects on plant productivity. Surprisingly, not many breeding programs are aimed at creating genotypes tolerant to **both** these types of stresses (e.g. salt and waterlogging tolerant). This may partially be due to the complex physiological nature of these stresses (Akhtar *et al.* 1998; Ashraf 2003; Barrett-Lennard 2003; Huang *et al.* 1995a) Waterlogging and salinity affect many physiological processes at all levels of organization from the whole plant level to molecular level (Gadallah 1999; Noble 1994). Identification of heritable traits is important for selecting for waterlogging and/or salt tolerance.

Reclaiming saline soils and halting the spread of this environmental thread is of great importance (Blacklow 2003; Cocks 2001; Ghassemi *et al.* 1995). To reduce the economic burden imposed by salinity and waterlogging on plant productivity, plants and genotypes best suited to these conditions will need to be identified. This will require a concerted research effort and understanding of the physiological mechanisms underlying plant tolerance to these stresses, as specific features of interactions between these two stresses. Also, in addition to being important from an agricultural point of view, the candidate species should at least be able to prevent further degradation of the farmland, if not ameliorate it. From this point of view, lucerne seems to be an ideal candidate.

Lucerne (*Medicago sativa*) is a deep-rooted perennial with a high potential rate of transpiration. Its taproot system can penetrate several metres deep into the soil profile and dewater the soil substrate. It has been successfully used in dryland salinity areas of Western Australia, where it has lowered the recharge by up to 150 mm over the course of one season (Humphries and Auricht 2001; Latta *et al.* 2001). However, like most other agricultural crops, lucerne is not very tolerant to the stress of waterlogging and salinity either alone or in combination. To increase the use of lucerne for the amelioration of degraded farmland, more tolerant

genotypes must be found. To determine lucerne's productivity and survival under dryland salinity conditions, some trials have been established. These include phase-farming trials, where lucerne is grown for 2-4 years in a crop rotation system (Latta *et al.* 2001; Latta *et al.* 2002) or overcropping trials of wheat with lucerne near Katanning in the south west of Western Australia (Humphries *et al.* 2004). Although results of these experiments and others (Crawford and MacFarlane 1995; Lolicato 2000) are very encouraging, all of them are empirically based. More knowledge about mechanisms, underlying salt and waterlogging tolerance in lucerne, is needed.

There have been few studies of the combined stresses of waterlogging and salinity (reviewed by Barrett-Lennard 2003) on crop plants such as wheat (Akhtar *et al.* 1998; Barrett-Lennard *et al.* 1999; Gadallah 1999), *Trifolium* species (Rogers and West 1993), squash (Huang *et al.* 1995a; Huang *et al.* 1995b) and grapes (West and Taylor 1984). However the physiological responses of lucerne to these stresses are poorly understood, and only few studies have investigated this (Barta and Sulc 2002; Rogers 2001; Rogers *et al.* 2003). One of the objectives of this project was to start to fill this knowledge gap.

Although responses to hypoxia and salinity are genetically and physiologically complex, genotypic variation of the stress response mechanisms in lucerne provides scope for selection and breeding of more tolerant lines. Screening of germplasm in the glasshouse for the initial selection of potentially tolerant breeding lines or individual plants is required to identify a promising pool of candidates that are adapted to the stresses of waterlogging and salinity. Developing appropriate screening techniques was another major objective of this project.

1. 2. OBJECTIVES AND RESEARCH AIMS

As mentioned above there are two major objectives to this research:

Elucidate the Underlying Mechanisms of Waterlogging and Salinity Tolerance in Lucerne

Different species have developed different adaptive mechanisms to abiotic stresses. Oxygen deficiency is likely the most important cause of injury in waterlogged plants (Kozlowski 1984a & b). However, some morphological and physiological adaptations may allow normal physiological processes to be maintained; for example aerenchyma formation helps maintain oxygen transport from shoots to roots (Colmer 2003a; Evans 2004), hypertrophied lenticels assist in gas exchange (Kozlowski and Pallardy 2002) and regeneration of new roots might influence the level of tolerance in different crop species (Garthwaite *et al.* 2003; Singh and Singh 2003; Voesenek *et al.* 1999). A low rate of radial oxygen loss (ROL) in rice is an adaptative feature that allows greater penetration of roots into anaerobic soils (Colmer *et al.* 1998). Stimulated shoot elongation during flooding is an important adaptive mechanism to restore contact of leaves with the air above the water surface. *Rumex palustris* will elongate its petioles in response to submergence (Voesenek *et al.* 2004) and rice is also able to reduce flooding stress by rapid elongation of its submerged tissues to keep up with the rising water (Vriezen *et al.* 2003).

Plants exposed to salty environments also face multiple constraints, e.g. osmotic stress, specific Na^+ or Cl^- toxicity, and various nutritional disorders (Greenway and Munns 1980). Different plant species have developed rather different strategies to deal with these constraints. For example, some plants are able to adjust their water potentials through accumulation of compatible solutes thus maintaining a water potential gradient between surrounding environment and plant tissue. Compatible solutes act as osmolytes to facilitate the retention of water in the cytoplasm and allow sequestering of sodium into the vacuole (Bohnert *et al.* 1995). Plants are classified as glycophytes or halophytes according to their capacity to deal with salt stress. Halophytic plants appear to have developed superior survival mechanisms and adaptations such as ion compartmentation, osmotic adaptations, succulence, selective transport and uptake of ions, and salt excretion (Adams *et al.* 1992; Clipson 1987). Glycophytic plants are also able to combat salinity to some degree by employing all or some of the

adaptive mechanisms listed above. There is, however, a wide spectrum of salinity tolerance mechanisms among halophytes (Flowers *et al.* 1977) as well as glycophytes (Maas *et al.* 1977; Shannon 1997), and most crop plants have only limited tolerance to the stresses of waterlogging and/or salinity. Usually salt can be prevented from entering the plant through its root system as well as be restricted from entering sensitive plant organs via internal exclusion mechanisms, eg by sequestering the harmful salts in the vacuoles. The compartmentation of salt in the vacuole protects proteins and membranes from ion toxicity. Salt excretion via salt glands and specialized bladder cells is another mechanisms available to halophytic plants to combat salinity. Crop plants, however, do not possess such specialized excretion tools. Plasma membranes are important sites of ion transport regulation. Numerous membrane transporters are known to be up- and down-regulated by salinity (Hasegawa *et al.* 2000; Koyama *et al.* 2001; Mansour *et al.* 2003; Tester and Davenport 2003), thus contributing to plant salt tolerance. What are the salt-tolerance mechanisms employed specifically by lucerne?

The ultimate test of tolerance to any abiotic stress is the plant's ability to maintain optimal biomass production under adverse conditions and recover promptly after removal of the stress. However responses vary throughout plant ontogeny and survival at germination does not necessarily mean survival at later stages of development or *vice versa*.

As previously stated, there are only few studies on the physiological responses of lucerne the combined effect of these abiotic stresses. Thus, **the overall aim was to better understand mechanisms of salt and waterlogging tolerance in lucerne**. Keeping in mind another major objective of this study (evaluation of potential tools for screening plants for tolerance), most of this work focused on the whole-plant level (e.g. stress-induced responses in growth, germination, photosynthesis, pigment analysis, chlorophyll fluorescence, and nutrient composition). Some insights into underlying cellular mechanisms (cell ultrastructure; electrophysiology) are also given.

The second major objective of this project was:

Evaluate Potential Screening Methods for Waterlogging and Salinity Tolerance in Lucerne

Among plant breeders, there is a persistent demand for developing quick, reliable and non-destructive methods of germplasm screening, to be used for screening hundreds and thousands of prospective genotypes. This is especially crucial for lucerne, which being an outcrossing species, shows a great degree of genetic variability (Al-Khatib *et al.* 1994; Musial *et al.* 2002). Therefore, the second objective of this work was to **evaluate potential screening methods for waterlogging and salinity tolerance in lucerne.** This work has been carried out as part of the ACIAR (Australian Centre for International Agricultural Research; Grant AS1/1998/026) funded project *Lucerne for Animal Production and the Environment*, in collaboration between Australia and China. It has as one of its premises the diagnosis of abiotic stress responses in lucerne and the development of a useful protocol of lucerne screening for waterlogging and salinity tolerance. To this end stress response characteristics were investigated and evaluated for their potential usefulness in a screening programme and possible avenues for plant improvement through selection and breeding are being proposed.

Soil type, topography and other locally determined parameters all exert some influence on the performance of lucerne (and other crops) under abiotic stress conditions in the field. Glasshouse experiments provide a controlled environment, where parameters such as temperature, light, nutrition and level of hypoxia or concentrations of salt treatment can be manipulated. Severity and duration of stress can be altered and closely monitored. Glasshouse trials are more cost efficient compared with field trials, which usually require a greater input of human resources due to their scale. Accuracy of field-testing results may also be compromised since many parameters fluctuate not only spatially but also temporally, during the time-course of experiments in the field. Therefore glasshouse trials lend themselves to detailed physiological studies as compared to field experiments, where achieving homogeneity of environmental parameters can be problematic. Although field trials are imperative for validation of test results (Setter and Waters 2003), glasshouse trials nonetheless may be useful for early screening efforts to eliminate genotypes, which are obviously poor performers and

test only those ones in further field trials, which are identified as relative tolerant to either or both stresses in the controlled environment.

1. 3. OUTLINE OF CHAPTERS

The thesis is divided into 10 chapters.

Chapter 1 is a general introduction in which the overall objectives are outlined.

Chapter 2 is a methodological overview as well as theoretical treatise of the screening methods and the tools used to identify stress tolerant plants.

Chapter 3 is a literature review on the topic of oxygen deprivation and the reactions and adaptations to waterlogging stress. It also identifies and highlights knowledge gaps with particular reference to lucerne.

Chapter 4 reports on my experiments into waterlogging stress responses in lucerne with the aim of identifying and validating a potential set of screening tools for waterlogging tolerance.

Chapter 5 (also experimental) characterises waterlogging recovery responses on the basis of chlorophyll fluorescence and nutrient dynamics. It aims to determine the level of recovery for chlorophyll fluorescence and other physiological parameters, to provide insight into potential genetic variability of lucerne recovery from waterlogging.

Chapter 6 is a literature review on salinity stress and summarizes major findings from the literature and identifies some knowledge gaps in relation to salinity stress in crop plants and in particular in lucerne.

Chapter 7 is focused on salt tolerance in lucerne and reports on germination studies comparing 10 different lucerne genotypes as well as some preliminary studies into the concurrent stresses of waterlogging and salinity.

Chapter 8 reports on experimental evidence for whole-plant and cellular aspects of salt tolerance mechanisms in lucerne.

Chapter 9 (also experimental) identifies ultrastructural changes to mesophyll cell structure due to salinity stress.

Chapter 10 summarises major findings reached in previous chapters and briefly discusses them in the context of the overall objectives. Overall conclusions drawn from the research are presented.

CHAPTER 2

SCREENING LUCERNE FOR TOLERANCE TO WATERLOGGING AND SALINITY: METHODOLOGIES AND THEORETICAL CONSIDERATIONS

2. 1. INTRODUCTION

Farmers aim to increase productivity of agricultural crops to maximize economic yield and thereby monetary returns. Environmental stresses threaten productivity of agricultural systems and can cause long-term deterioration of valuable land, which poses a serious threat to food security. Genetic improvement is one way of achieving increased productivity, particularly in adverse conditions such as waterlogged and saline environments.

Tolerance to waterlogging and salinity by higher plants, in common with other environmental stresses, is genetically and physiologically complex, and oxygen and salt stress affect numerous plant processes at all levels of organization. At the cellular level, membrane selectivity and ion transport patterns across both internal and external membranes are affected by salinity (Amtmann and Sanders 1999; Blumwald 2000; Maathuis and Amtmann 1999; Tester and Davenport 2003). Oxygen stress affects membrane integrity through ATP deprivation and reduction of lipid synthesis (Rawyler *et al.* 2002). At the whole-plant level, plant growth, net CO_2 assimilation, water use efficiency, leaf photochemistry, stomatal conductance and pigment composition are other physiological parameters influenced by salinity (Chen *et al.* 1999; Delfine *et al.* 1999; Koyama *et al.* 2001; Lawlor and Cornic 2002) and waterlogging (Armstrong and Drew 2002; Jackson and Drew 1984; Visser *et al.* 2003).

Linking physiological and genetic information is a major objective of modern plant breeding (Flowers *et al.* 2000). Characterizing physiological stress responses in lucerne, therefore, is an important step in the process of genetic

improvement in salt and waterlogging tolerance of lucerne. In this chapter I identify several potential indicators of stress tolerance and discuss their possible advantages and disadvantages.

2. 2. SCREENING

Plant breeders have used visual traits to screen germplasm for desirable attributes and identify individuals, expressing these traits. If genes imparting salinity tolerance for example were tightly linked to a visible trait, screening would be easy. Lack of suitable screening techniques to identify salt and waterlogging tolerant lucerne genotypes and the time-consuming and expensive classical breeding programmes make it necessary to look for quicker and more sensitive methods of screening.

As mentioned before, both salt- and waterlogging-tolerance have a polygenic nature. Expression of these genes may have synergistic or antagonistic effects. These are also referred to as *quantitative genes* and do not follow Mendelian gene inheritance patterns. Genes that condition the expression of quantitative characters are called, "quantitative trait loci", in short QTL (Falconer and Mackay 1996; Haussmann *et al.* 2004; Maloof 2003). Their expression is difficult to score. Thus it is unfeasible to select individuals merely by visual observations. Moreover, to obtain lines with all the desired genes (contributing to the traits of interest) combined in non-segregating individuals is (a) labor intensive, (b) environment dependent, and (c) time consuming because selection of individuals in advanced generations needs to be carried out on a large population.

Flowers *et al.* (2000) cautioned against the use of marker assisted selection for physiological traits, but instead highlights the need for direct knowledge of the genes conferring tolerance. However, identifying the gene for a particular character from the huge amount of DNA within an organism is a voluminous task, and it becomes more complicated in the case of polygene-controlled quantitative traits like resistance to abiotic stresses.

The ultimate criterion for developing tolerant genotypes must be the ability to maintain near optimal productivity under saline and waterlogged conditions in the field. However, the field environment is too variable in which to undertake initial selection. Initial selection will need to be under controlled waterlogging and salinity conditions in the glasshouse, where most environmental parameters can be adjusted to the experimental design. Eventual validation needs to occur later in the field (Setter and Waters 2003). So far, no suitable method has been developed for lucerne. The establishment of lucerne with tolerance to hypoxia and salinity requires the development of quicker and more sensitive methods for identifying individuals with desirable attributes from a large, genetically diverse population.

One of the easiest and obvious tools for screening might be by assessing changes in plant growth characteristics and biomass production. Other screening tools, which might prove effective, include pigment concentrations, photosynthetic characteristics, chlorophyll fluorescence, anatomical changes to cell structure and cell organelles and membrane transport processes in conjunction with other parameters such as growth characteristics.

2. 3. PLANT BIOMASS PRODUCTION

The process of photosynthesis results in the conversion of light energy to drive chemical reactions, most notably the reduction of carbon dioxide to carbohydrates. Biomass is one important indicator of a crop's capacity for this conversion. High yielding agricultural crops ought to have high photosynthetic rates and energetically efficient dark respiration (Hall 1993). The aim for agricultural crops is to maintain overall photosynthetic capacity, i. e. high assimilatory rates and quantum yields under adverse conditions. Abiotic stresses are likely to influence the conversion efficiency of light energy to chemical energy. Measuring biomass production is one aspect of studying plant productivity responses to external stresses (Munns and James 2003; Musgrave 1994) that can be used to validate early physiological, non-destructive stress indicators.

Optimal biomass production and yield in adverse conditions are the ultimate measure of the relative tolerance level of crops grown in waterlogged and saline conditions. Therefore monitoring biomass production under stress is a useful screening tool to identify genotypes or individuals, which display a greater degree of tolerance. However, screening germplasm for biomass or yield is very time consuming and may extend the duration of experiments considerably, in the case of measuring grain yield for example. Furthermore it is an integral parameter, which does not give any clues about the underlying mechanisms. Quicker methods, with reliable predictability need to be identified.

2. 4. PHOTOSYNTHETIC PARAMETERS

2. 4. 1. Introduction

Photosynthesis provides the basis for biomass production since it drives the cycling of CO_2 uptake and O_2 evolution. Capacity of CO_2 assimilation depends on diffusional resistance and this may be influenced by abiotic stresses. CO_2 dissolves intracellularly and diffuses into chloroplasts, where it reaches the carboxylating enzyme Rubisco (Atwell *et al.* 1999). Understanding photosynthetic mechanisms from the ecosystem level down to primary reaction centres is important to appreciate how abiotic stresses such as waterlogging and salinity might compromise its functioning (Mostowska 1997; Shabala 2002). Improving plant productivity in environmentally challenging circumstances requires a basic understanding of photosynthetic processes in order to optimise conditions for optimal growth. Photosynthesis is fundamental to plant productivity, but many factors such as light and temperature as well as other abiotic stresses modify its level.

2. 4. 2. CO_2 Assimilation

Measurement of CO_2 assimilation not only provides a sensitive indicator of short-term growth of crops, but it is also a method for identifying environmental and genotypic differences in photosynthetic capacity. Many environmental stresses lead to a decrease in the efficiency of light energy

conversion (Mostowska 1997; Taiz and Zeiger 1998). In particular, photo-inhibitory damage to the photosynthetic mechanism due to anaerobiosis can be expected.

While photosynthetic mechanisms appear to be relatively stable at low salinity levels, higher concentrations of NaCl will cause damage to the photosynthetic apparatus and will lead to yield reductions (Bethke and Drew 1992; Everard *et al.* 1994; Ma *et al.* 1997). Stomatal conductance and hence CO_2 assimilation can be significantly affected at intermediate salinity levels. Hence, photosynthetic gas exchange analysis can indicate responses of carbon fixation to salt stress at intermediate to high (50-300 mM NaCl) salinities.

Photosynthetic energy conversion pertains to the whole photosynthetic process from light capture to CO_2 assimilation and its subsequent metabolism in the chloroplast. To understand how efficiency of this process may be improved a greater understanding of the photosynthetic process is necessary.

Photosynthetic gas exchange analysis can give an indication of the responses of carbon fixation to abiotic stresses in the environment and identify genotypic responses and potentially highlight tolerant plants. Summary tables of the use of gas exchange parameters for assessing effects on waterlogging and salinity are given in the review Chapters 3 and 6.

2. 4. 3. Stomatal Conductance

Stomata play an important role in controlling the balance between water loss and carbon gain, i.e. biomass production. To reduce water loss, plant leaves have an epidermal layer with a relatively impermeable cuticle and turgor-operated valves called stomata. The epidermis reduces CO_2 and water vapour exchange and also controls assimilation and transpiration through its pores. CO_2 used in leaf photosynthesis gains entry via stomata. Leaves control this gas exchange by adjusting the aperture of stomata (Atwell *et al.* 1999). Stomatal conductance can be influenced by abiotic stresses such as anaerobiosis or salt (Ashraf 2003; Huang *et al.* 1995a; Tattini *et al.* 1999). It is believed that at intermediate salinity levels the predominant mechanism affecting net CO_2 assimilation is stomatal limitation,

while non-stomatal limitations are a significant factor at higher salinities (Bethke and Drew 1992; Seemann and Critchley 1985; Delfine *et al.* 1998; Di Martino *et al.* 2003; James *et al.* 2002). Stomatal conductance has been measured in screening trials to assess effects of abiotic stresses such as waterlogging and salinity (Anand *et al.* 2000; Ashraf 2003; Belkodja *et al.* 1999; Chen *et al.* 1999; Huang *et al.* 1995a; Smith and Moss 1998; Wagner and Dreyer 1997). Generally it has been found that stomatal conductance is reduced by the stresses of waterlogging and salinity on their own and more so if they occur concurrently (Ashraf 2003). Since measurement of stomatal conductance is non-destructive it might prove to be a good technique to determine differential flood tolerance between species and genotypes.

Stomata provide regulated gas exchange in plants, which is affected during both waterlogging and salinity. Measuring the effect of abiotic stresses on stomatal conductivity should improve our understanding of water use and photosynthetic efficiency and identify tolerant genotypes.

2. 4. 4. Pigment Analysis

Measurement of chlorophyll *a* and *b* concentration can potentially provide some indication of potential stress effects of anaerobiosis and/or salinity on plant photosynthetic performance and, ultimately, plant yield. In case of salinity stress this parameter may however be somewhat problematic as there are some reports of increased pigment concentration (Mäkelä *et al.* 2000; Winicov and Seemann 1990) at least initially, before salinity stress causes chlorosis. At the same time, salt-induced chlorophyll reduction occurred in rice and clear genotypic responses between tolerant and sensitive cultivars were observed at 30 and 50 mM NaCl treatment after one week and became accentuated after three weeks of stress (Lutts *et al.* 1996). James *et al.* (2002) also found cultivar responses in wheat of differing tolerance level. The sensitive cultivar Woolaroi showed a greater degree of leaf injury assessed on the basis of chlorophyll concentration. Chlorophyll per unit leaf area was reduced in *Phaseolus vulgaris* L. (Seemann and Critchley 1985); in *Gossypium* hybrids only the salt sensitive one showed a significant reduction in chlorophyll content.

Waterlogging has been shown to cause increased chlorosis with increasing duration of oxygen stress (Ashraf and Habib-ur-Rehman 1999; Daugherty and Musgrave 1994; Huang *et al.* 1994; Webb *et al.* 1996). Huang's *et al.* (1995a) study on squash revealed depressed chlorophyll production in waterlogged as well as saline squash. The effect on pigment concentration was exacerbated when both stresses occurred concurrently, but partial recovery of pigment concentration after free drainage as well as after removal of saline stress was observed.

Several reports suggest genotypic differences in pigment concentration as a result of abiotic stresses (see above); hence pigment analysis may well prove to be a suitable screening tool, especially in concurrence with other physiological parameters. This method however remains to be validated for lucerne.

2. 5. CHLOROPHYLL FLUORESCENCE

2. 5. 1. Theoretical Basis

The light energy absorbed by chloroplasts first excites pigment molecules of the light harvesting chlorophyll proteins (LHC). These LHC proteins transfer their energy to either Photosystem I (PSI) or Photosystem II (PSII). These photosystems contain the reaction centre pigments for the conversion of absorbed light energy to oxidation and reduction potential to drive dark electron transport (Genty *et al.* 1989; Maxwell and Johnson 2000; Waldhoff *et al.* 2002).

Photons are transferred as excitons to the reaction centres where they are finally trapped by a pair of chlorophyll a molecules (P680). Such traps are regarded as open if Q_A is fully oxidised and ready to receive an electron from P680. If however, Q_A is still in its reduced state, that "trap" will be closed and excitons will then transfer to another reaction centre or be lost as heat or fluorescence. Chlorophyll fluorescence from PS II is thus diagnostic of changes in the functional state of the photosystem (Atwell *et al.* 1999).

Upon absorption of light, the excited antenna chlorophyll *a* molecules have several potential pathways for de-excitation: (1) energy transfer to other chlorophyll *a* molecules as part of the resonance energy transfer, (2) deactivation as heat and (3) re-emission as fluorescence (Kautsky and Hirsch 1931; Maxwell

and Johnson 2000; Rohácek 2002). Under normal conditions, 80-90% of the absorbed light energy is photo-chemically converted; chlorophyll fluorescence comprises 1-3% and the rest is lost as heat. Under stress conditions, however, ratios will change dramatically and the non-photochemical components will rise (Jimenez *et al.* 1997; Lichtenthaler 1988; Schreiber and Bilger 1987; Schweiger *et al.* 1996; Van Kooten and Snel 1990).

Changes in fluorescence yield reflect changes in photochemical efficiency and heat dissipation (Maxwell and Johnson 2000). The variable part of chlorophyll fluorescence originates mainly in photosystem II. Measurements of stress-induced changes in the ratio of photochemical to non-photochemical components of fluorescence will give an indication of the extent of damage. Chlorophyll fluorescence is usually considered as a non-invasive optical tool with high diagnostic value (Bolhar-Nordenkampf and Oequist 1993; Krause and Weis 1991).

Upon illumination with a sufficiently strong light (saturation light pulse), fluorescence increases from Fo via an intermediate level (I) and often a dip (D) to a peak (P) level. All reaction centres are closed (Q_A is fully reduced) and the saturating light briefly suppresses photochemical yield to zero and induces maximal fluorescence (Fm). The rise reflects a surge of electrons, which fill successive pools of various electron acceptors of PS II and the rate of photochemistry concurrently declines (Bolhar-Nordenkampf and Oequist 1993). A useful index derived from these parameters is the so-called variable fluorescence (Fv), which equals the fluorescence increase from Fo to Fm. The scale and kinetics of this rise are significantly influenced by all kinds of environmental stressors. The Fv/Fm ratio measured after dark treatment reflects the proportion of efficiently working PS II units among the total PS II population.

Thus fluorescence is a measure of the photochemical efficiency of a leaf and it correlates well with other measures of photosynthetic effectiveness. Plants of diverse taxa, habitats and leaf structure with well developed and fully functioning photosynthetic apparatus typically have values of Fv/Fm around 0.83;

this is proportional to the quantum yield of photochemistry (Björkman and Demmig 1987).

Two types of fluorescence quenching processes must be considered in order to understand the relationship between photosynthesis and fluorescence. Photochemical quenching (qP) is determined by photochemistry so that photochemical quenching decreases in proportion to the closure of the reaction centres, i.e. reduction of Q_A. Non-photochemical quenching (qN) is mainly determined by de-excitation through heat generation, and its largest contributor is the energy dependent quenching created by the pH gradient across the thylakoids (Schreiber and Bilger 1987; Schreiber *et al.* 1994; Schreiber *et al.* 1995). Minor components of non-chemical quenching are controlled by the amount of excitation energy transferred to PS I (Bolhar-Nordenkampf and Oequist 1993).

In order to determine maximal photochemical efficiency of PS II expressed as Fv/Fm the leaf must be dark adapted for at least 30 minutes to reverse all non-photochemical fluorescence quenching; an alternative is to take readings pre-dawn. A decline in Fv/Fm is a good indicator of photoinhibitory damage during stress. Its use as a screening tool to assess stress effects has been widely advocated (Belkodja *et al.* 1994; Nogues *et al.* 1994; Shabala 2002).

2. 5. 2. Chlorophyll Fluorescence as a Sensitive Stress Indicator and Screening Tool

Changes in the overall efficiency of leaf photochemistry can be detected by a change in chlorophyll fluorescence. This includes all the reactions from the reduction of water through to electron transport, development of the electrochemical gradient, ATP synthesis, and eventually the series of enzymatic reactions for CO_2 reduction to carbohydrate in the leaf (Maxwell and Johnson 2000; Rohácek 2002). The function of thylakoid membranes is sensitive to several environmental stresses such as high temperatures, draught, freezing, waterlogging and excessive radiation (Sayed 2003; Schreiber and Bilger 1987; Schweiger *et al.* 1996; Shabala 2002). All theses stresses affect the function of PS II directly or indirectly and therefore chlorophyll fluorescence can be used to reveal stress response mechanisms such as the efficiency of energy conversion or the degree of

reaction centre closure in photosystem II (Baker 1991; Greer and Halligan 2001; Lichtenthaler 1996). Even if the mechanistic background of stress reactions is not fully understood, chlorophyll fluorescence measurements can serve as a screening tool for individual plants to select plants with a greater level of stress tolerance (Bolhar-Nordenkampf and Oequist 1993).

Crop growth and yield is highly dependent on the efficacy of the photosynthetic machinery. Environmental stresses such as drought and waterlogging inhibit photosynthesis in wheat (Lu and Zhang 1998). Measuring chlorophyll fluorescence is a convenient non-intrusive method to monitor photosynthetic events at different functional levels (Krause and Weis 1991). One of the major advantages of the chlorophyll fluorescence technique as a screening tool in plant breeding is the ability to screen hundreds of plants per day following a relatively simple sampling protocol. Another major advantage is that the technique is non-destructive and therefore selected individuals can subsequently be used for further breeding or clonal propagation (Shabala 2002). It is important to remember that consistent criteria be employed with any technique (chlorophyll fluorescence is no exception). Selection of the leaf type and age to be measured has to be consistent so that only results from comparable measurements are analysed.

Although chlorophyll fluorescence has been widely used to measure stress responses to various environmental stresses (Bolhar-Nordenkampf and Draxler 1993; Fracheboud *et al.* 1999; Jimenez *et al.* 1997; Kriedemann *et al.* 1985; Lichtenthaler 1996; Lu and Zhang 1998; Maxwell and Johnson 2000; Mohammed *et al.* 1995; Ralph 2000; Schreiber *et al.* 1994; Shabala 2002), there are only a handful of reports about its use in studies related to the abiotic stress of oxygen deprivation (Haldimann and Strasser 1999; Smethurst and Shabala 2003; Wagner and Dreyer 1997). Summary tables of the use of chlorophyll fluorescence for assessing effects of waterlogging and salinity are given in the review Chapters 3 and 6. There is no published material on chlorophyll fluorescence dynamics in stress conditions in lucerne.

2. 6. ANATOMICAL CHANGES OF CELLULAR AND SUB-CELLULAR STRUCTURES

NaCl induces oxidative stress in chloroplasts causing membrane damage and other structural alterations (Hema *et al.* 2003; Parida *et al.* 2003). Thylakoidal structure of chloroplast was notably disorganized in the NaCl-treated leaves of *Pisum sativum*, and number and size of plastoglobuli increased (Hernandez *et al.* 1995). It is suggested that NaCl toxicity such as superoxide- and H_2O_2-mediated oxidative damage in chloroplasts may play an important role in membrane disorganization. Adaptations to environmental stresses in the thylakoid membranes are accompanied by corresponding changes in the chloroplast arrangements within the cell (Li and Ong 1997; Md. Shahidur *et al.* 2000; Parida *et al.* 2003; Sam *et al.* 2003). Mesophyll cell tissue is also likely to change in response to stress; both palisade and spongy mesophyll may change as a consequence of environmental stresses. Salinity-induced increased vacuolation might lead to increased cell size (Mitsuya *et al.* 2000, also see Chapters 7 and 9), which has also been recorded in parenchyma of salt-stressed tomato (Sam *et al.* 2003).

Transverse sections of the leaf blades can reveal changes in leaf tissue architecture due to abiotic stresses and potentially identify different adaptive mechanisms by different genotypes. Transmission electron micrographs can illustrate structural changes at the cellular and sub-cellular level due to externally imposed stresses (Kurkova *et al.* 2002; Liang 1998; Md. Shahidur *et al.* 2000; Rahman *et al.* 2002). Thus, it was logical to assume that changes in the size of palisade parenchyma cells and/or chloroplast arrangement and shape may be indicative of the degree of salt tolerance in lucerne.

As a potential screening tool, determination of anatomical and cell ultra-structural changes are time consuming and therefore economically not very attractive, however they potentially provide a better understanding of the mechanisms at play and are able to shed light on genotypic differences at the cellular and sub-cellular level. To our knowledge there is no information on ultrastructural changes in salt-stressed lucerne.

2. 7. LEAF OSMOTIC POTENTIAL

Two principal adverse effects of salinity in non-tolerant plants are osmotic stress and specific ion (predominantly, Na^+) toxicity (Greenway and Munns 1980; Tester and Davenport 2003). In overcoming the osmotic stress each plant is facing the Scylla-Charybdis dilemma: to use Na^+ as a "cheap" osmoticum (Amtmann and Sanders 1999; Shabala and Lew 2002), or to synthesise intracellular compatible solutes required for osmotic adjustment (Bohnert and Sheveleva 1998; Serrano *et al.* 1999). Both these options lead to a significant increase in cell osmolality, in a process called osmoregulation or osmotic adjustment (Morales *et al.* 2002; Nakamura *et al.* 2002; Netondo *et al.* 2004). Cell sap osmolality, expressed as mole of solute per kg of water can be measured relatively easily by determining the change in freezing point compared to deionized water. Therefore, it is not surprising that osmotic potential measurements of cell sap revealed genotypic differences in responses to salt stress (An *et al.* 2002; Nakamura *et al.* 2002; Netondo *et al.* 2004; Qian *et al.* 2001) and were suggested as a useful indicator of salt stress tolerance. A decrease in leaf osmotic potential due to waterlogging was also recorded for sugarcane, kiwi fruit and almond (Roberts *et al.* 1990; Sanchez-Blanco *et al.* 1994; Save and Serrano 1986) and genotypic variation was observed for almond and kiwi fruit. Hence cell sap osmolality in lucerne may prove to be a useful indicator for stress with the potential of revealing genotypic differences.

2. 8. MEMBRANE-TRANSPORT CHARACTERISTICS

2. 8. 1. Membrane Transporters and Plant Adaptive Responses

Plant membranes constitute a barrier to free diffusion of molecules and are believed to be the primary sites of perception of virtually every known environmental stress (Zimmermann *et al.* 1999). Membrane transport and uptake processes are integral to ion and solute movement and therefore important targets for characterizing adaptive mechanisms. Pumps, channels, and carriers are all part of the ion transport mechanisms across membranes (Amtmann and Sanders 1999). Both salt stress (Blumwald 2000; Hasegawa *et al.* 2000; Maathuis and Amtmann 1999; Tester and Davenport 2003; Tyerman and Skerrett 1999) and hypoxia (Felle

1996; Leul and Zhou 1999; Rawyler *et al.* 2002) cause significant changes in ion transport activity across the plasma and organelle membranes. To prevent or alleviate nutrient imbalances and deficiencies caused by salt stress plants must re-establish homeostatic conditions (Zhu 2001). Multiple mechanisms may confer plant salt tolerance at the membrane-transport level, including restriction of Na^+ from uptake (Amtmann *et al.* 2001; Demidchik and Tester 2002; Rus *et al.* 2001; Tester and Davenport 2003; Tyerman *et al.* 1997), active Na^+ extrusion via plasma membrane H^+/Na^+ antiport mechanism (SOS1) (Hasegawa *et al.* 2000; Qi and Spalding 2004; Zhu 2003), Na^+ sequestering into vacuole via tonoplast H^+/Na^+ exchanger (NHX) (Apse *et al.* 1999; Serrano and Rodriguez-Navarro 2001), and maintaining optimal K^+/Na^+ ratio via regulation of K^+ transport across the plasma membrane (Maathuis and Amtmann 1999; Shabala 2003).

Various methods can be employed to study changes in membrane-transport activity in plant cells under stress conditions. That may include destructive sampling and depletion experiments (Barber 1995; Garnett and Smethurst 1999; Kelly *et al.* 1992), use of radioactive tracers (Rengel 1996; Schachtman and Thomas 2003; White *et al.* 1991), nuclear magnetic resonance (NMR) technique (Rokitta *et al.* 2004), fluorescence microscopy (Roos 2000), patch-clamp technique (Maathuis and Prins 1990; Roberts and Tester 1997), traditional ion-selective electrodes (Hinke 1987), impaled microelectrodes (Carden *et al.* 2003) and non-invasive ion flux measurements (Babourina *et al.* 2000; Demidchik *et al.* 2003; Newman 2001; Shabala *et al.* 2003; Shabala *et al.* 1997). A comprehensive analysis of these methods is given by (Shabala L 2002), and the latter two methods are further characterised in relation to salinity studies in the following sections.

2. 8. 2. Microelectrode Impalement Techniques

Traditionally, microelectrode impalement technique has been first applied to measure voltage gradients between both sides of the plasma membrane, so called membrane potential (MP). In a "typical' plant tissue, including most root epidermis and leaf mesophyll, MP values are in the range of –100 to –150 mV (cytosol negative) (Elzenga *et al.* 1995; Kochian *et al.* 1992; Lew 1998; Shabala

2000). Most stresses, including salinity and waterlogging, cause significant depolarisation of these values. Membranes depolarized in salinized potato suspension cells (Hawkins and Lips 1997). *Ceratopteris richardii* also responded with membrane depolarization when exposed to salt stress (Warne *et al.* 1996). Hypoxia induces membrane depolarisation and K^+ loss from wheat roots (Buwalda *et al.* 1988) and beetroot storage tissue (Zhang *et al.* 1992). As transport of all nutrients is directly or indirectly linked to MP values, the more significant the membrane depolarisation, the more severe is the disturbance to cell ionic homeostasis. Thus, when comparing membrane potential measurements across a pool of genotypes, differences may potentially indicate varying levels of salt stress or waterlogging tolerance. Therefore, membrane potential measurements can be expected to identify tolerant individuals in a genetically diverse population, if membrane potential dynamics are indeed genetically determined.

A more sophisticated method involves a microelectrode tip being filled with a specific ionophore, sensitive to a particular ion (e.g. Na^+ or K^+) (Carden *et al.* 2001; Pineros *et al.* 1998; Wells and Miller 2000). As the reference electrode also has to be impaled alongside with the ion-selective microelectrode, multi-barrelled electrodes are often used for these purposes (Carden *et al.* 2003). This technique thus makes it possible to monitor changes in cytosolic ion homeostasis and therefore provide answers on some fundamental questions about ionic mechanisms underlying stress tolerance in plant cells. However, being extremely technically- and skill-demanding, this technique is barely applicable as a potential screening tool. Instead, the non-invasive Microelectrode Ion Flux Estimating technique (MIFE) was used in this study.

2. 8. 3. Non-Invasive Ion Flux Measurements

A novel non-invasive ion-flux measuring (MIFE) technique was introduced in plant physiology about 10 years ago. This technique was developed at the School of Physics, University of Tasmania (see Newman 2001 for review and background information). This technique involves the slow square-wave movement of ion-selective microelectrode probes between two positions, one close and the other distant from the cell surface. If an ion is taken up by the living

cell, its concentration in the proximity of the cell surface will be lower than some distance away. *Vice versa*, if the ion is extruded across the plasma membrane, there will be a pronounced concentration gradient directed away from the cell surface. The magnitude of the gradient will be proportional to the rate of ion movement across the plasma membrane, i.e. to the net flux of the specific ion of interest. The voltage gradients between the two positions are recorded and converted into concentration differences using the calibrated Nernst slopes of the electrodes. The advantage of this technique is its high temporal (5 s) and spatial (a few microns) resolution, a possibility to measure fluxes of several ions concurrently, and the unique feasibility for long-term (several hours) measurements without any significant impact on the organism studied (Shabala and Knowles 2002; Shabala *et al.* 1997). What appears to be the most important aspect of the MIFE technique is that it is **non-invasive** and thus some individuals can be retained after initial screening and grown on once identified as being tolerant. All this makes the MIFE technique a potentially valuable screening tool.

Over the last 15 years, the non-invasive ion flux measuring technique has been widely used to address plant responses to various types of abiotic stresses such as salinity (Babourina *et al.* 2000; Shabala 2000; Shabala *et al.* 2003), pH (Babourina *et al.* 2001; Shabala *et al.* 1997), osmotic stress (Shabala *et al.* 2000; Shabala and Lew 2002; Shabala and Newman 1998), chilling (Shabala and Shabala 2002; Shabala and Newman 1997), wounding (Hush *et al.* 1992; Meyer and Weisenseel 1997), Al^{3+} toxicity (Ryan and Kochian 1993; Ryan *et al.* 1992), and oxidative stress (Demidchik *et al.* 2003). Thus, it was expected that investigating basic ion flux kinetics in lucerne roots or leaves will shed more light on how various lucerne genotypes cope with environmental stresses such as salinity and/or waterlogging.

CHAPTER 3

PLANT RESPONSES TO OXYGEN DEPRIVATION – STRESS REACTIONS AND ADAPTATIONS

3. 1. INTRODUCTION

In many parts of the world – including Australia and China – agricultural production is adversely affected by heavy seasonal rainfall and poorly drained soils (Armstrong and Drew 2002). It is estimated that about 10% of the global land area is affected by severe soil drainage constraints. However, in certain areas such as Eastern Europe and the Russian Federation, the estimate is closer to 20% (Setter and Waters 2003). Waterlogging can be endemic or be the result of human intervention such as in Australia where clearing native vegetation has lead to rising water tables (Blacklow 2003).

In well-drained soils, air-filled pores make up 10%-60% of the total soil volume, allowing O_2 and CO_2 to diffuse freely around plant roots (Moore 1998). However, when soils become saturated and all airspaces are filled with water, O_2 supply decreases rapidly. The low solubility of oxygen in water (0.28 mol m^{-3} at 20° C) and the low diffusivity of oxygen in water-filled pores, i.e.10 000-fold slower than through gas-filled pores (Armstrong and Drew 2002; Grable 1966), as well as the rapid consumption of dissolved oxygen by bacteria and roots all contribute to hypoxia and ultimately anoxia (Barrett-Lennard 2003; Ponnamperuma 1984). The time it takes to totally deplete oxygen depends on soil temperature (Drew 1983; Rogers 1974) and the respiring biomass present (Armstrong and Drew 2002). This makes oxygen shortage one of the major abiotic stresses that determines the success or failure of many agricultural crops grown in Australia as well as in other parts of the world.

Understanding how plants are affected physiologically and what adaptive mechanisms are employed to combat the stress is of importance economically and ecologically especially in light of degrading agricultural environments globally.

As well as causing physical and chemical changes in the soil, waterlogging directly affects some key physiological characteristics such as respiratory metabolism, water and nutrient uptake by roots. The functioning of the photosynthetic apparatus is compromised in waterlogged plants (Huang *et al.* 1997; Malik *et al.* 2001). Oxygen can diffuse from aerial parts to the roots, but the amounts thus transported are usually not sufficient for the oxygen requirements of agricultural crops and other dryland plants. In wetland plants, internal oxygen transport can contribute significantly to root respiration under anaerobic conditions (Colmer 2003a; Laan *et al.* 1990). Wetland species also seem to possess a specialized metabolism, in addition to morphological changes, that allows them to gain sufficient energy when there is not enough molecular oxygen to act as the terminal electron acceptor for cytochromes.

The imbalance between production and consumption of assimilates under waterlogged conditions is ultimately the cause of reduced growth under hypoxic stress. When gas exchange is impeded survival may be prejudiced by creating a short-fall in the energy needs for maintaining cell integrity in key tissues such as root tips and meristematic regions of leaf bases and stems (Jackson and Ram 2003).

Susceptibility to the stress of waterlogging can vary between species and even between cultivars of one species (Huang *et al.* 1994; Marcar *et al.* 2002; Mustroph and Albrecht 2003). Tolerance depends on many plant specific as well as environmental factors. Waterlogged plants have a range of coping mechanisms to enhance survival, including morphological adaptations in roots such as adventitious root growth, formation of aerenchyma, and hypertrophied lenticels. Metabolic adaptations include altered nutrient uptake mechanisms to cope with the nutrient stress commonly found under hypoxic conditions. Maintenance of cytosolic pH and membrane integrity is crucial for the survival of anoxic conditions (Greenway and Gibbs 2003). Temperature, light regimes, soil pH, and

organic content influence the success or otherwise of surviving periods of hypoxia (Kozlowski 1984a).

Plant adaptive mechanisms ensure at least temporary survival in low O_2 conditions, but aerial growth and final crop yield may be compromised. Oxygen deficiency in the soil may lead to ultimate plant death if the period of stress is either too severe or too sustained. When aerobic conditions are restored before lethal effects have occurred physiological functions can usually be recovered (Barrett-Lennard *et al.* 1988; Malik *et al.* 2002).

Plant stress responses and adaptive mechanisms of hypoxia and anoxia have been studied extensively and many thorough reviews on various aspects of oxygen deficiency symptoms and tolerance mechanisms have been published. There are several recent reviews on signal transduction (Dat *et al.* 2004; Jackson 2002) and the role of ethylene in flooding resistance (Voesenek *et al.* 1992). Blokhina *et al.* (2003) presented a detailed account of the generation of reactive oxygen species (ROS) due to oxygen deprivation and the damage to lipids, proteins, carbohydrates, and nucleic acids. Specific aspects of energy production and distribution in relation to anoxia tolerance have been recently reviewed by Greenway and Gibbs (2003) and Gibbs and Greenway (2003). Armstrong and Drew (2002) provided a comprehensive review on root growth and metabolism under oxygen deficiency with special emphasis on morphological and biochemical adaptations. Other papers and reviews by Colmer (2003b); Evans (2004), Geigenberger (2003); Vartapetian and Jackson (1997) have elucidated further aspects of waterlogging stress. Setter and Waters (2003) demonstrated the importance of considering environmental factors for germplasm improvement for waterlogging tolerance in grain crops, while Barrett-Lennard (2003) focused on the effects of the interaction of waterlogging and salinity on ion relations, growth and survival in higher plants.

Despite this significant attention towards the understanding of the mechanisms underlying plant waterlogging tolerance, many unanswered question remain, such as species specific response characteristics, development of stress responses over time and the physiological effects of severity and duration of

waterlogging. Certain plant species have been studied quite thoroughly, for example rice (Jackson and Ram 2003; Kirk 2003; Rubinigg *et al.* 2002; Vartapetian and Polyakova 1999); wheat (Huang *et al.* 1994; Malik *et al.* 2001; Sharma and Swarup 1989; Stieger and Feller 1994; Trought and Drew 1980) and maize (Ashraf 2003). Studies on lucerne in relation to waterlogging, however, are scarce (Barta and Sulc 2002; Zook *et al.* 1986).

In this review I summarize some major findings related to biochemical, nutritional and morphological responses to waterlogged and other oxygen deficient environments, in an attempt to identify knowledge gaps in this field.

3. 2. BIOCHEMICAL ALTERATIONS

The key factor in waterlogged soils is the change from oxidizing to reducing conditions due to anaerobic respiration by soil bacteria. As soon as soil is flooded and all pores are filled with water, gas exchange between the soil and the atmosphere is restricted (Ponnamperuma 1984; Voesenek *et al.* 1992). Root oxygen deficiencies in flooded soils limit extension growth. As well as that it initiates stress protein production, byproducts of anaerobic metabolism, lipid peroxidation (Blokhina *et al.* 2003), hormonal changes, leakiness of solutes and ultrastructural changes in mitochondria (Vartapetian and Jackson 1997; Gibbs and Greenway 2003; Greenway and Gibbs 2003).

3. 2. 1 Soil Redox Potential and Iron and Manganese Toxicity

Oxygen in soil is chemically reduced to water by the terminal step in the respiratory electron transport chain of microorganisms and plant roots. After complete oxygen depletion, anaerobic microorganisms use various oxidized soil components as electron acceptors, leading to a sharp decline in soil redox potential (Voesenek *et al.* 1992).

Aerobic soil micro-organisms use the oxygen present in the soil. When O_2 supply is depleted, CO_2 and other gases produced in respiration and alcoholic fermentation accumulate (Moore 1998). The activity of anaerobic microbes is stimulated. Soil redox potential can become very low (-200mV) and toxic forms

of microelements such as iron and manganese (Fe^{2+} and Mn^{2+}) develop (Ponnamperuma 1972). For example soil redox potential reached -100mV after only four days of waterlogging (Stieger and Feller 1994). At this value roots suffer from O_2 deficiency and reduction of manganese and iron becomes significant. Organic matter is anaerobically decomposed, which produces organic acids that are highly toxic to plants particularly at low pH. In O_2 starved soils, reduced Fe and Mn are solubilised and may become toxic for crop plants (Stieger and Feller 1994). The increased availability of these ions in soil affects delivery to the plant, which is likely to lead to higher Fe and Mn concentrations in plant tissue (Ashraf and Rehman 1999). The adsorption and desorption behaviour of P was affected by Fe oxides in rice (Zhang and Lin 2003). Although Fe and Mn concentrations can reach supra-optimal range, they are often not high enough to cause toxicity symptoms (Colin-Belgrand *et al.* 1991; Sharma and Swarup 1989; Barta 1988; Rogers 1974). Results of Mn and Fe dynamics in waterlogged soil are not conclusive, however, and further studies are required to elucidate the role of these ions in hypoxic conditions.

3. 2. 2. ATP Production – Reduced Energy Supplies

Roots normally require oxygen for optimal production of adenosine triphophate (ATP) from sugars. Under aerobic conditions glucose in roots is oxidized to 6 mol of CO_2 and 6 mol of H_2O to produce up to 38 mol of ATP. Under anaerobic conditions, glucose is oxidized to produce 2 mol of ethanol and 2 mol of CO_2 with a yield of only 2 mol of ATP. The significant decrease in ATP production has consequences for plant metabolism in waterlogged soil, since ATP is the energy required to fuel nearly all cellular processes (Barrett-Lennard 2003).

Cell death of flooded roots may be due to the fact that anaerobic respiration produces insufficient ATP for growth and cell maintenance. Probably all plant cells are able to survive anoxia for periods of some hours, during which ATP has to be generated by anaerobic metabolism (Armstrong and Drew 2002). Even if metabolism is slowed in anoxic cells, ATP will still be required for maintenance and for synthesis of various metabolites, including the proteins that are synthesized anaerobically.

Root survival under anoxia can be extended by exogenous supplies of carbohydrate suggesting that cells soon use up their supplies of easily-respired substrates, while further translocation of substrates from shoot to root are presumably curtailed (Webb and Armstrong 1983).

3. 2. 3. Signal Transduction and Hormonal Responses

Coordinated growth of shoots, roots and other organs depends on an interchange of signals (hormones and other signalling agents/molecules); however, the coordination on a whole plant basis is poorly understood (Jackson 2002). Waterlogging triggers cellular signal transduction pathways leading to physiological and morphological changes. O_2 deficiency is probably the primary signal triggering plant and soil responses (Dat *et al.* 2004). Changes in solute fluxes out of the root and into the shoot due to hypoxia can also be interpreted as biochemical signals.

The critical hormone involved in plant response to waterlogging appears to be the ethylene. Jackson's (2002) review on long–distance signalling in waterlogged plants highlighted the importance of ethylene production, entrapment and action in oxygen deficient roots and associated signals to target cells in the shoots to affect such responses as epinastic leaf curvature, stomatal closure and slowing of leaf extension. Ethylene production is accompanied by a signalling cascade, which induces a network of hormones and other common secondary signalling molecules (Dat *et al.* 2004). Hypoxia leads to modification of ethylene biosynthesis. Ethylene has been implicated in hypoxia-induced aerenchyma formation and evidence indicates that ethylene production is necessary for cell death in hypoxic roots of maize (Morgan *et al.* 1997; Drew *et al.* 2000). Lack of oxygen results in a rapid inhibition of oxidative phosphorylation in mitochondria and therefore a decrease in cellular ATP, an increase in NADH, and changes in a number of cellular metabolites (Blokhina *et al.* 2003).

Other major plant hormones (abscisic acid, auxin, cytokinins, and gibberellins) are also involved in mediation of waterlogging signalling. Flooding is known to depress cytokinin and gibberellin activity in xylem sap (Neuman *et al.* 1990). These negative signals may enhance epinasty response since applying

exogenous cytokinin and gibberellin can inhibit epinasty (Jackson 2002). Decreased leaf growth is a particular strong and prompt response to flooding (Else *et al.* 1996).

Cross-talk between ABA and ethylene in regulating growth has recently been suggested by Sharp *et al.* (2000). The large decrease in ABA delivery that takes place after flooding might sensitise leaves to the action of ethylene (Jackson 2002). Delivery of ABA in xylem sap is reduced (Else *et al.* 1994), but foliar ABA concentrations are often increased and are implicated in stomatal closure (Jackson and Hall 1987).

Other hormonal interplay has also been described during flooding stress. Synergism between indolylacetic acid (IAA), and ethylene has been proposed during adventitious root formation (Dat *et al.* 2004). The increase in ABA in leaves in waterlogged plants is well documented but the process by which ABA levels increase is less clear. It has been suggested that loss of hydraulic conductance induces ABA production in leaves (Jackson 2002). Raised ABA concentrations provide a ready explanation for a decline in stomatal conductance and leaf growth (Armstrong and Drew 2002). Stomatal regulation is sensitive to the ABA concentrations in the apoplast of adjoining guard cells.

Not only hormones but also various inorganic ionic species such as hydrogen ions, nitrate or calcium may function as signalling molecules (Jackson 2002). Transient changes in the concentration of cytosolic Ca^{2+} are part of a signal transduction pathway triggered by anoxia in maize roots (Subbaiah *et al.* 1994). The rapidity with which Ca^{2+} changes during anoxia suggests that it responds to the drop of cytoplasmic pH or to lowered energy metabolism. The signalling pathway between increased Ca^{2+} and anaerobic protein production remains to be elucidated (Drew 1997). The role of Ca^{2+} during aerenchyma development has been suggested (Drew *et al.* 2000). It appears that Ca^{2+} plays a role in the signalling cascades during hypoxia gene regulation and aerenchyma formation (Dat *et al.* 2004).

3. 2. 4. Enzymes of Glucose Phosphate

One of the early responses to oxygen deprivation is the induction of genes encoding enzymes of glucose phosphate metabolism, such as alcohol dehydrogenase, enolase, and glyceraldehydes phosphate dehydrogenase (Saab 1999). Because mitochondrial respiration is inhibited by oxygen deprivation, the induction of these enzymes permits limited energy production by glycolysis and fermentation and, thus, aids in short-term survival of flooding. Accelerated glycoysis during anoxia (Pasteur Effect) can occur, however, even when high rates of glycolysis happen, plant cells produce at most 37 % of the ATP produced under aerated conditions, but usually much less (Greenway and Gibbs 2003).

3. 2. 5. Avoidance of Cytoplasmic Acidosis

Control of metabolically generated protons is critical to survival of anoxia. Formation of metabolites, such as succinate, has been proposed as a means of consuming excess H^+ during anoxia (Menegus *et al.* 1989; Roberts *et al.* 1992), thereby offsetting cytoplasmic acidosis. Formation of gamma-aminobutyric acid (GABA) from glutamate has also been suggested as a means of consuming protons and delaying acidosis (Reid *et al.* 1985). Decarboxylation of malic acid to yield pyruvate, through the low pH activation of malate dehydrogenase appeared to be consuming a significant quantity of protons during the early response to anoxia with pyruvate subsequently converted by transamination to alanine (Edwards *et al.* 1998).

Cell death under anaerobic conditions is closely associated with acidification of the cytoplasm. Acidification of the cytoplasm is caused by leakage of protons from the vacuole, which is normally maintained at around pH 5.8 compared with pH 7.4 of the cytoplasm. Lowering of ATP within the cytosol reduces the proton pumping capacity of ATPases while H^+ leaks passively to the cytoplasm (Armstrong and Drew 2002). A rapid drop of cytoplasmic pH with anoxia has been confirmed in root hairs of *Medicago sativa* using pH sensitive microelectrodes (Felle 1996). Regulation of cytoplasmic pH is central to cell survival.

3. 2. 6. Hypoxic Pre-Treatment

Maintaining the energy status within viable cells during anoxia can be achieved in part by the ability to continue glycolysis, fermentation and ATP synthesis (Armstrong and Drew 2002).

Hypoxic pre-treatment improves tolerance to anoxia (Chang *et al.* 2000; Hole *et al.* 1992) and was associated with the ability to maintain a greater rate of glycolysis and ethanolic fermentation. Hemoglobin is an important oxygen binding protein, occurring in most living cells. A mild hypoxia pre-treatment (5%) induced the gene for increased hemoglobin and increased the survival of Arabidopsis after severe hypoxic treatment (Hunt *et al.* 2002). Hypoxic pre-treatment resulted in a smaller decrease of cytosolic pH (Xia and Roberts 1994), which reduced energy costs for maintenance. Greenway and Gibbs (2003) postulated that hypoxic pre-treatment of anoxia tolerant tissue induces acclimative changes in addition to the increase in potential for the rate of ethanolic fermentation.

3. 2. 7. Carbohydrate Metabolism

Root apices represent the most metabolically active sites in roots and have the lowest levels of carbohydrate reserves (Barta 1988). Assimilate transport to the roots and metabolites to structural components were significantly decreased for both waterlogged lucerne and birdsfoot trefoil; root soluble sugars were significantly increased in response to flooding, root starch also increased under anoxia (Barta 1987). Perennial forage legumes such as lucerne accumulate large quantities of starch in their roots, however, starch does not appear to be readily utilized as a carbohydrate source under anoxia (Barta 1987).

Waterlogging can also affect the pattern of source-sink interaction in whole plants. Carbohydrate transport to roots and subsequent phloem unloading were both reduced in many species under hypoxic conditions (Barta 1987; Stieger and Feller 1994). Soluble sugars were higher in sap of waterlogged chickpea (Cowie *et al.* 1996) and lucerne plants (Barta 1988). Starch concentrations were

also higher in both stem (Cowie *et al.* 1996) and leaf (Vu and Yelenosky 1991) tissues of waterlogged plants.

Assimilate supply during waterlogging affects recovery potential of chickpea (Cowie *et al.* 1996) and lucerne (Barta 1988). In depleted oxygen conditions, nutrient reserves are limited and need to be mobilized from storage tissues such as the stem (Cowie 1996).

3. 2. 8. Implications for Lucerne

Little is known about the biochemical dynamics in waterlogged lucerne, with no targeted studies on ATP production, signal transduction and hypoxic pre-treatment in waterlogged lucerne species. The only related work was published by Felle (1996) who studied cytoplasmic pH regulation in *Medicago sativa* root hairs. However, as avoidance of cytoplasmic acidosis and membrane integrity are major adaptive mechanisms to oxygen deficiency, it can be expected lucerne to behave similarly to other dryland cropping plants with regard to maintaining homeostasis as a means of avoiding cell disintegration.

Future development of improved waterlogging resistance in lucerne may be through engineering an efficient anaerobic metabolism to help the root system tolerate more extended periods of life transiently without oxygen.

3. 3. MORPHOLOGICAL AND ANATOMICAL ADAPTATIONS OF ROOTS AND SHOOTS

3. 3. 1. Adventitious Roots

Developing adventitious roots is a mechanism for replacing existing roots that have been killed or whose function is impaired due to oxygen deficiency (Vartapetian and Jackson 1997). Such roots are developing near the water or soil surface where O_2 concentrations are highest and where the roots are more likely to encounter oxygen either directly from the surroundings or translocated internally through aerenchyma (Justin and Armstrong 1987). Hence the ability to survive in anaerobic soils may not be due to a high resistance of root cells to anoxia but instead be expressed by the ability of re-directing root elongation laterally or

upwards to reach regions where oxygen is not limiting (Vartapetian and Jackson 1997). The role of adventitious roots includes supplying water, minerals and hormones and acting as sinks for shoot assimilates and metabolites.

For example, flooded oak roots decayed, while hypertrophied lenticels and subsequently adventitious roots developed on the taproot of different oak species (Colin-Belgrand *et al.* 1991). Rapid decay of pre-existing roots including senescence and disappearance of white tips (growing root apices) and necrosis of the taproot and lateral roots were common root growth responses to flooding observed in oak; adventitious roots developed after 4 weeks of flooding (Colin-Belgrand *et al.* 1991). In wheat, Huang *et al.* (1994) reported significant inhibition of root biomass production in both tolerant and intolerant cultivars of wheat, but the tolerant variety initiated vigorous adventitious roots that proliferated abundantly and thoroughly exploited the rooting medium.

Waterlogging arrested the growth of seminal roots in wheat and aerenchymatous nodal roots grew and extended into the anoxic solution but only to a length of about 12 cm and only few were produced (Trought and Drew 1980c). On resuming aeration nodal roots elongated, lateral roots on the seminal roots also resumed extension, but apical meristems of the main seminal roots were moribund. Baruch (1994) attributed relative waterlogging tolerance of tropical forage grasses to their ability to form hollow stolons, which enhanced oxygen diffusion to the roots and the development of adventitious rootlets, which promoted greater nutrient absorption. Hence, adventitious roots are believed to replace the functions of the original roots and help in the survival and recovery after waterlogging.

3. 3. 2. Aerenchyma Formation

The ability to form a continuum of gas space between the root cortex, the shoot and the atmosphere via the stomata and lenticels is an important adaptation that helps prevent or alleviate oxygen deficiency in roots. Aerenchyma formation enhances survival and sustains basic cell functions and structures, presumably as a result of improved oxygenation (Vartapetian and Jackson 1997). For most species the ability to withstand soil waterlogging depends on the capacity to transport

oxygen internally at a volume and speed that averts root anoxia (Colmer *et al.* 1998; Thomson *et al.* 1990). Aerenchyma permit internal oxygen transport; this may still not be sufficient to maintain aerobic respiration; it depends largely on the root length, root porosity, root radius, stellar radius, oxygen consumption of the soil and the root respiration rate (Voesenek *et al.* 1992). However, some plants may never form aerenchyma (Armstrong and Drew 2002).

Justin and Armstrong (1987) demonstrated that aerenchyma are capable of delivering oxygen in up to 300 mm long roots, despite losses en route from radial leakage and respiration and thereby enhancing survival and sustaining basic root cell functions and structures. Aerenchyma not only promote long distance transport of oxygen to the roots but also facilitate the counter-flow of volatile compounds accumulated near the root; these include ethanol, CO_2 and methane (Vartapetian and Jackson 1997). Concentration gradients are needed to drive the oxygen transport from shoots to roots via aerenchyma. The gradients are created by oxygen consumption in the roots (Armstrong 1979).

However, deep-rooted plants (such as lucerne), which are suddenly exposed to waterlogging, are unlikely to be able to benefit form aerenchyma formation. Rather aerenchyma development will benefit plant communities which are exposed to regular cycles of waterlogging or plants with short roots or those that are able to form adventitious roots rapidly (Barrett-Lennard 2003).

In many species, development of aerenchyma starts with the death of cells in the root cortex. Cell death then progresses radially and tangentially into surrounding cells (Justin and Armstrong 1987). Low partial pressures of oxygen initiate aerenchyma formation. In anoxia, on the other hand, the process is arrested and the tissue remains intact, until death by necrosis occurs (Evans 2004). For oxygenation over greater distances, aerenchyma are necessary and effective since resistance to gas diffusion and convection is lowered. Partial disintegration of root tissue not only allows for internal gas transport but also lessens the amount of respiring tissue per unit root volume and therefore consuming less O_2 (Drew 1997).

3. 3. 4. Suberization and Cell Wall Thickening to Prevent Radial Oxygen Loss (ROL)

Wetland species have roots with lignified and suberised secondary cell walls that develop within a few mm of the root tip. These structures help conserve O_2 by reducing permeability and thus preventing its radial loss to the surrounding medium (Drew 1997). ROL apparently increases with distance behind the root tip in terrestrial plants such as *Trifolium tomentosum* and *T. glomeratum,* but decreases with distance behind the root tip in wetland plants such as rice due to a physical barrier to oxygen diffusion in the epidermis of the root (Gibberd *et al.* 1999).

Suberized roots may conflict with the need for efficient nutrient absorption, however (Kirk 2003). This author postulated that different root tissues in rice take on different functions; the laterals, which are plumbed directly into the main water and solute transport vessels, are responsible for the bulk of nutrient absorption and compensate for any impairment of nutrient absorption by the primary roots. Primary roots on the other hand are adapted for internal aeration and cell walls are largely impermeable to gases, thus reducing oxygen loss. Wall thickening in the hypodermal layer induced by waterlogging might not only be important in the conservation of oxygen, but at the same time resisting phytotoxin entry and damage (Armstrong and Drew 2002; Rubinigg *et al.* 2002). Albrecht and Mustroph (2003) suggested that cellulose deposits also provide mechanical reinforcement of the root tissue.

3. 3. 5. Enhanced Shoot Elongation

In cases of partial submergence, it is important for petioles and/or internodes to maintain or indeed enhance their elongation rate to achieve and maintain contact with the atmosphere via the shoot to gain access to atmospheric oxygen (Voesenek *et al.* 1992). Promotion of shoot elongation by submergence is known to occur in wetland species over a wide range of species reviewed by Jackson and Armstrong (1999). It constitutes an escape mechanism from asphyxiation by submergence. During the short duration of flash floods, elongation may prove fatal, however, because it competes with maintenance

processes for energy and hence may reduce survival (Setter and Laureles 1996) and it can lead to an imbalance between production and consumption of assimilates (Jackson and Ram 2003). Fast elongation under water constitutes a high risk strategy when flooding events are of short duration, because when flood waters subside elongated petioles can become brittle and weak and can desiccate rapidly after desubmergence (Voesenek et al. 2004). Also, young rice plants may not be able to reach beyond the water level to make contact with the air, thus causing the young rice plant to die (Jackson and Ram 2003).

3. 3. 6. Oxygenation of Rhizosphere

Oxygenation of the rhizosphere by radial leakage of oxygen from aerenchyma can be of importance because it oxidatively de-toxifies chemically reduced iron, manganese and hydrogen sulfide and may support nitrifying bacteria that convert ammonia to nitrate (Kirk 2003; Vartapetian and Jackson 1997). Gibberd *et al.* (1999) showed that *Trifolium* plants responded to hypoxia by increasing root porosity measured as increased volume of cortical airspaces. ROL formed a sink for O_2 over the entire length of the root that in turn decreased the maximum possible length of these roots in waterlogged soils (Armstrong 1979).

3. 3. 7. Shoot and Leaf Adaptations

Premature senescence of older leaves is significantly higher in waterlogged than in control plants of rapeseed (Boem *et al.* 1996) as well as in wheat grown in hypoxic solution culture (Trought and Drew 1980c). Leaf epinasty was observed in the less tolerant tropical grasses as well as leaf rolling (Baruch 1994). Trought and Drew (1980a) also observed tightly rolled leaves on emergence in wheat, which only unrolled when elongation was nearly complete. Leaves elongating during waterlogging, were slower to do so and were ultimately shorter than control leaves. Inadequate soil aeration caused wilting of leaves since hydraulic conductivity of the whole root system declined rapidly. Oxygen deficient roots also regulate shoot processes such as growth and senescence as well as influence shoot water relationships via the transpiration stream. Many stresses, including flooding, slow transpiration by promoting stomatal closure.

3. 3. 8. Specifics of Morphological and Anatomical Adaptation in Lucerne

Lucerne has a very wide range of adaptation in part due to its extensive taproot system. Root depth to 18 m in Russian soil have been recorded (Kutschera 1960); more commonly lucerne taproots reach length of 3-4 m, that allow the extraction of water from deep in the soil profile.

Apart from this fact, not much is known however, about morphological adaptations to waterlogging in lucerne. Zook *et al.* (1986) was the first to report aerenchyma formation in flooded lucerne. Studies into the variation of morphological responses in lucerne are needed to identify possible superior genotypes. McDonald *et al.* (2002) claimed that the prospect of using genotypes that show superior aerenchyma formation are promising with regard to enhancing these traits in waterlogging intolerant crops. We observed adventitious root growth in lucerne together with the disintegration of the taproot after prolonged flooding (Smethurst and Shabala 2003). A search for superior germplasm, which is capable of withstanding prolonged waterlogging, on the basis of morphological adaptations is warranted.

3. 4. NUTRIENT DYNAMICS

Waterlogging and oxygen deficiency can directly influence nutrient uptake by affecting the availability of nutrients in the soil solution and by changing root activity (Stieger and Feller 1994; Morard and Silvestre 1996). Altered source-sink relations within the shoot can also influence the redistribution of phloem-mobile nutrients from older, senescing leaves to new growth (Boem *et al.* 1996). Several publications in relation to nutrient dynamics in hypoxic conditions are catalogued in Table 3. 1.

3. 4. 1. Underlying Cellular Mechanisms

The link between oxygen supply and ion transport is believed to be principally through respiration and the generation of ATP to drive transport (Zhang and Greenway 1995). Anaerobic metabolism does not maintain energy supplies at a level that will drive active transport via the H^+-translocating ATPase

in the plasma membrane. As a result, the regulation of both cytoplasmic pH and membrane potential are impaired (Greenway and Gibbs 2003), thus affecting solutes movement across membranes. After five hours of hypoxia the membrane potential of beetroot cells dropped from –156mV to –95mV (Zhang *et al.* 1992), while tissue electrical conductivity increased, indicating a loss of membrane permeability (Morard and Silvestre 1996). Upon re-aeration red beet cells hyperpolarized to values more negative than those in continuous air (Zhang and Greenway 1995). Thus, it is generally accepted that efflux of major cations from plant roots observed under hypoxia conditions is due to depolarization of root cell membranes rather than increases in the permeability of membranes to specific ions (Buwalda *et al.* 1988; Greenway and Gibbs 2003). However, it is also important to keep in mind that a reduced transpiration flux under anaerobic conditions reduces ion transport to the shoot by mass flow.

3. 4. 2. Basic Macronutrients

Inhibited ion uptake by roots and transport to shoots in waterlogged soil is commonly observed (Armstrong and Drew 2002; Boem *et al.* 1996; Drew 1988; Singh *et al.* 2002). Consequently, plants grown in O_2-depleted soils show a marked decrease in the rate of accumulation of most macronutrients in above ground plant tissues (Boem *et al.* 1996; Sharma and Swarup 1989). A comparable inhibition of ion transport to shoots occurs also in deoxygenated nutrient solutions (Morard and Silvestre 1996; Trought and Drew 1980c).

In the short term N, P and K are more affected than Ca^{2+} and Mg^{2+} (Trought and Drew 1980b), reflecting active versus passive uptake (Ashraf and Rehman 1999). These authors also reported that N, P and K were translocated from older leaves to younger, growing leaves and this was associated with premature senescence. Over longer periods of anaerobiosis, passive uptake mechanisms of minerals such as Ca^{2+} and Mg^{2+} are also disturbed (Morard and Silvestre 1996). So far, little (if any) difference in nutritional status between tolerant and susceptible cultivars has been found (Lizaso *et al.* (2001). While most authors are unanimous in their estimation of waterlogging effects of leaf

nutritional status, there appears to be a great deal of controversy about nutrient dynamics in waterlogged roots.

Ashraf and Rehman (1999) found N, P, and K in corn leaf tissue decreased as a result of flooding for 21 d, whereas the reverse was true for the roots. Sharma and Swarup (1989), however, reported decreases of N, P, and K in both shoots and roots of wheat, and attributed reduced tillering mainly to decreased P uptake. Obviously, more studies are required to resolve this issue.

3. 4. 3. Nitrogen

Decreases in N contents are often an early response to flooding. These decreases may either be due to nitrate reduction and accelerated denitrification or due to the inability of roots to take up enough N even before the onset of strong denitrification (Colin-Belgrand *et al.* 1991).

Nitrate can be translocated via the transpiration stream while ammonium is generally assimilated in the roots. Organic substrates delivered from the shoot to the roots are required during ammonium utilization to fuel assimilatory processes and to provide the C skeletons for the synthesis of organic compounds transported via the xylem to the shoot. Flooded soils can have high concentrations of NH_4 (Stieger and Feller 1994). If N is predominantly in the form of NH_4, much of the plant's energy production goes into carbon metabolites for the incorporation of NH_4 and its detoxification. This phenomenon results in diversion of energy and carbohydrates away from growth thereby reducing plant growth (Ashraf and Rehman 1999). Trought and Drew (1980b) found that addition of foliar nitrogen application in the form of urea-N or application of nitrate and ammonium to the soil had some beneficial effect on waterlogged plants such as slightly increased chlorophyll concentration and an increase in fresh weight of the shoot.

3. 4. 4. Micronutrients

A large increase in dissolved and exchangeable manganese and iron in the soil solution after waterlogging was observed (Stieger and Feller 1994). As a result the contents of iron and manganese in the plant shoots were higher in waterlogged soil. Fe increased both in leaves and roots but Mn content in shoots

and roots was not affected by waterlogging (Stieger and Feller 1994; Chorianopoulou and Bouranis 2004). Zn and Cu concentrations increased under oxygen deficiency in the wetland species *Apium nodiflorum* and it was hypothesised that this may be related to an enhanced reactive oxygen species detoxification system, as both Zn and Cu are included in the structure of superoxide dismutase, which detoxifies superoxide anion (Chorianopoulou and Bouranis 2004).

Table 3. 1. Effects of waterlogging on nutrient concentrations in plant tissue (w/l = waterlogged)

Species	Treatment	Plant Part	Unit	Control (aerated)				Waterlogged				Reference
				N	P	K	Ca	N	P	K	Ca	
Brassica napus	7 d w/l	Shoot and seeds	mmol g^{-1} DW	46.8	2.2	16.2	7.7	22.8	1.4	9.1	6.1	Boem *et al.* 1996
Hordeum vulgare	14 d N_2 bubbled nutrient solution	Shoots	mmol g^{-1}DW			1.72				1.36		John *et al.* 1977
Medicago sativa	14 d w/l	Roots	µmol g^{-1} DW			15.7				11.4		Barta 1988
Quercus robur	7 weeks w/l, 60 mm below soil surface	Leaves	g kg^{-1} DW	33.4	1.7	8.23	9.47	26.6	1.28	6.6	8.32	Colin-Belgrand *et al.* 1991
Triticum aestivum	15 d w/l in sandy soil	Shoot	µmol g^{-1} DW	2016	2	864	95	621	23	259	68	Trought and Drew 1980a
Triticum aestivum	31 d w/l field trials	Grains	µg/spiklet	27.48	325	512.5	56		158.3	312.5	31.7	Stieger and Feller 1994
Zea mays	21 d w/l sandy loam	Leaves	g kg^{-1} DW	2.23	1.71	21		13.18	1.42	19		Ashraf and Rehman 1999
Zea mays	6 d w/l sandy loam	shoots	% DW		0.5	3.48	0.55	1.33	0.31	1.15	0.44	Lizaso *et al.* 2001

3. 5. OTHER FACTORS INFLUENCING PLANT RESPONSES TO WATERLOGGING

3. 5. 1. Plant Ontogeny

The stage of plant development (Boem *et al.* 1996) is an important factor determining severity of stress responses, including those to waterlogging (Cowie *et al.* 1996). Increased sensitivities to waterlogging at certain stages of growth (such as flowering stage; Cowie *et al.* 1996) complicate experimental design and interpretation. In general flooding during the growing season is much more harmful than flooding when plants are dormant (Kozlowski 1984a & b).

3. 5. 2. Soil Temperature

The rapidity with which waterlogging sensitive plants become stressed is largely dependent on ambient temperature, because chemical changes with rising temperature associated with oxidation and reduction of the soil environment are speeded up. Gases that are consumed, like O_2, will be rapidly depleted and gases which are produced, like CO_2 and ethylene will accumulate more rapidly at higher temperatures (Drew 1983). In lucerne, soil temperature greatly affected its susceptibility to flooding (Rogers 1974), as it could withstand longer periods of flooding when the root zone temperature was reduced from 25°C to 19°C (Heinrichs 1970). Higher air and soil temperatures caused a more severe response in lucerne to soil waterlogging (Cameron 1973). In wheat, the rate of respiration in response to increases in temperature is linear between 9°C and 20°C (Morard and Silvestre 1996). Yield reductions in rapeseed were more severe in Boem's *et al.* (1996) investigation, compared to the experiment of Cannell and Belford (1980) where soil temperatures were about 5°C lower.

3. 5. 3. Timing and Duration of Waterlogging

The highly variable nature of waterlogging both in space and time highlight the complexity of possible mechanisms of recovery. Short term or intermittent waterlogging requires plants to maintain survival strategies, while growth is of secondary importance. Strategies such as high rates of fermentation

to overcome energy deficiency during anoxia, high carbohydrate concentrations to sustain alcoholic fermentation, maintenance of membrane integrity and reduced metabolite leakage, increased efficiency of nutrient uptake, and decreased damage to O_2 free radicals associated with the return to aerobic conditions are all traits characterizing tolerance to short-term or intermittent periods of waterlogging (Setter and Waters 2003). Tolerance to long-term waterlogging events requires plants not only "survive" but also grow during the waterlogging event. One of the key strategies employed for long-term waterlogging stress is the development of aerenchyma in roots to facilitate gas diffusion (Blom 1999; Jackson and Armstrong 1999). Other adaptations to long-term waterlogging include suberisation of adventitious roots to provide a barrier to radial O_2 loss (Colmer 2003b).

3. 6. WATERLOGGING AND PHOTOSYNTHESIS

3. 6. 1. General

One of the most prominent symptoms of plant stress due to waterlogging is the development of chlorosis, signalling a degradation of the photosynthetic apparatus (Zhou and Lin 1995).

Photosynthesis is the central anabolic pathway in plants for the production of organic compounds necessary for growth. Functional disorders of the photosynthetic electron transport and CO_2 fixation are induced by oxygen deficiency. Changes in photosynthesis, the loss of photosynthetic capacity, the degradation of the photosynthetic apparatus and the accumulation of inactivated PSII centres can in part be understood as a process of adaptation to a decreased demand of assimilates (Godde 1999; Bohnert *et al.*1995; Drew 1983, Kozlowski 1984a). Slowed anaerobic carbohydrate catabolism leading to decreases in the rate of glycolysis may be due to its downregulation or feedback inhibition (Gibbs and Greenway 2003; Setter and Waters 2003). Prerequisites for sustained energy production during anoxia are substrate availability and a mechanism, which avoids accumulation of endproducts, so that potential accumulation of endproducts will not result in toxicity, such as cytoplasmic acidosis (Gibbs and

Greenway 2003). Feedback inhibition from accumulation of soluble carbohydrates can lead to a lowering of the photosynthetic rate and cause damage to photosystem II reaction centres.

3. 6. 2. Chlorophyll Content, Stomatal Conductance and CO_2 Assimilation

Hypoxia has adverse effects on net photosynthesis (P_n), stomatal conductance (g_s), water relations, and leaf chlorophyll content (Huang *et al.* 1997). Inhibition of photosynthesis by waterlogging is attributed to decreases in leaf water potential and stomatal conductance (g_s), reduction in photosynthetic enzymes, and inhibition of photosynthetic transport resulting from reduced sink demand and decreases in chlorophyll content (Huang *et al.* 1994; Malik *et al.* 2001; McFarlane *et al.* 2003) and may also be influenced by waterlogging induced nutrient imbalance (see Table 3. 2.).

Vu and Yelenosky (1991) reported that continuous soil flooding of citrus caused a severe decline in photosynthetic rate, stomatal conductance and chlorophyll concentrations, as well as senescence, wilting and abscission of leaves. Chlorophyll declined rapidly after 7 days of continuous flooding in wheat (Thomson *et al.* 1992). Stomatal closure of flooded plants results from a hormonal signal transmitted from the roots to the leaves, possibly root-synthesized ABA. Flooding induced rapid increases of ABA in leaves and stomatal closure and accumulation of ABA are well correlated (Kozlowski and Pallardy 2002).

Rate of photosynthesis decreased in rice, wheat and maize (Mustroph and Albrecht 2003) with duration of hypoxia, in lucerne (Smethurst and Shabala 2003), in lupins (Davies 2000b), in eucalypts (Farrell *et al.* 1996), wheat (Malik *et al.* 2001) and many others (Gadallah 1999; Guidi and Soldatini 1997; Huang *et al.* 1995a; Stevens 1994; Wagner and Dreyer 1997).

Table 3. 2: Effect of waterlogging on photosynthesis

Species	Treatment	Major findings	Reference
Zea mays L.	waterlogged for 21d	Photosynthetic rate (P_n) reduced; stomatal conductance (g_s) reduced.	Ashraf and Habib-ur-Rehman 1999
Lupinus luteus	waterlogged for 14 d and recovered for 14 d	Photosynthetic rate (P_n) reduced; stomatal conductance (g_s) reduced.	Davies *et al.* 2000b
mungbean	waterlogged for 8 d at vegetative and reproductive phase	Photosynthetic rate, transpiration, diffusive resistance all significantly reduced between 60-80%, but able to recover within days.	Ahmed *et al.* 2000
Lolium perenne L.	waterlogged up to 28 d	Photosynthesis reduced by between 30 –50% after 28 d; genotypic variation in the response to waterlogging.	McFarlane *et al.* 2003
Quercus robur *Quercus petraea* *Quercus rubra*	waterlogging for 2-4 weeks; two levels of irradiance	net CO_2 assimilation, stomatal conductance, photosynthetic capacity and chlorophyll content declined.	Wagner and Dreyer 1997
Sultana grapevines	waterlogged for up to 7 d	Photosynthesis and stomatal conductance were significantly reduced by 20 and 40% respectively both during and following waterlogging.	Stevens and Prior 1994
Rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mays*)	Hypoxia or anoxia treated for 4 d either with N_2 bubbled nutrient solution (hypoxia) or nutrient solution and air flushed with nitrogen (anoxia)	In anoxic treatment wheat and maize reduced rate of photosynthesis by more than 70% and 90% respectively, rice remained at nearly 50% of control. In hypoxic treatment no significant changes in photosynthetic rate in all plant species investigated.	Mustroph and Albrecht 2003
Wheat *(Triticum aestivum* L.)	waterlogged to several different depth (0, 100 mm, 200 mm) for 14 d then recovered for 14 d	Net light-saturated rates of photosynthesis in leaves of plants growing in soils waterlogged to the surface reduced after 1 d and reduced more severely with increasing time up to a 70-80%; waterlogging to 200 mm below soil surface caused little reduction in net photosynthesis; stomatal conductance was similarly affected by different waterlogging depth.	Malik *et al.* 2001

3. 6. 3. Chlorophyll Fluorescence

In recent years, chlorophyll fluorescence has become a widely used tool in plant screening for environmental fitness (Shabala 2002; Mohammed *et al.* 1995; Schreiber *et al.* 1994; Lichtenthaler 1988). Measuring chlorophyll fluorescence is a convenient non-destructive method to monitor photosynthetic events at different functional levels, from pigment level to enzymatic stroma reactions and regulatory feedback processes (Genty *et al.* 1989; Maxwell and Johnson 2000). As photosynthetic machinery is sensitive to all sorts of environmental pressures, it is not surprising that fluorescence signals are used widely to assess the functioning of the PS II activity under various abiotic stresses such as heat, chilling, salinity, and to a lesser extent, waterlogging, to name a few. Over the last decade, hundreds of papers have been published on the use of chlorophyll fluorescence to monitor plant responses to these three types of stresses. However, the use of the chlorophyll fluorescence technique in research on waterlogging are rather limited (Shabala 2002; see also Table 3. 3.).

Wagner and Dreyer (1997) found that maximal quantum efficiency (Fv/Fm) was reduced after imposing waterlogging stress on oaks. Webb and Fletcher (1996) reported a similar response in wheat and Percival *et al.* (1998) in Italian elder (*Alnus cordata*). Exposure to prolonged anoxia caused a significant decrease of Fv/Fm from 0.83 in controls to 0.40 in waterlogged American cranberry (*Vaccinium macrocarpon*) (Schlueter and Crawford 2003). This was mainly attributed to reduced Fm levels, while Fo only showed a slight increase. A reduced level of Fv/Fm in the anoxia treated leaf indicated that the activity of the PSII reaction centres was impaired under anoxia. The reduction in maximum fluorescence Fm signified a decrease in the energy trapping of PSII, which might have been caused by denaturation of proteins (Schlueter and Crawford 2003). Photochemical quenching in anoxia treated cranberries was also impaired, signalling damage to electron transport.

Table 3. 3: Effect of waterlogging on chlorophyll fluorescence parameters

Species	Treatment	Major findings	Reference
Boltonia decurrens (wetland plant)	rootzone flooding up to 10 weeks	No effect on fluorescence ratio Fv/Fm; stomatal conductance more sensitive.	Smith and Moos 1998
Camptotheca accuminata	waterlogged up to 4 weeks; different light regimes	Significant reduction in Fv/Fm in full sun and light shade; no reduction in Fv/Fm under heavy shade.	Liu *et al.* 1997
Helianthus annuus	waterlogged for 11 d	Dark-adapted for 20 minutes; Fv/Fm unaffected.	Guidi and Soldatini 1997
Medicago sativa	waterlogged for 15 d	Predawn values of Fv/Fm decreased significantly and steadily over time to just below 0.75 (control 0.83); decline in Fm; increase in NPQ.	Smethurst and Shabala 2003
Quercus robur *Quercus petraea* *Quercus rubra*	waterlogging for 2-4 weeks; two levels of irradiance	Predawn values of Fv/Fm decreased significantly; decline of predawn values of photochemical efficiency was not as great in shade plants; species differences observed.	Wagner and Dreyer 1997
Soja hispida (soybean)	waterlogged for 13 d	Dark-adapted for 20 minutes; increase in Fo after 1 day, after that significant decline, no significant changes in Fv/Fm.	Guidi and Soldatini 1997
Triticum aestivum	waterlogged, 30 mm above soil surface, up to 5 weeks at different growth stages	Dark-adapted for 30 minutes; Fv/Fm values decreased by 10-18% - indicative of photoinhibitory damage.	Webb and Fletcher 1996
Vaccinium macrocarpon (cranberry)	anoxia induced in anaerobic chamber (90% N and 10 % H); plants kept in dark for up to 28 d; watered with N_2 bubbled water; recovery of up to 10 d	Anoxia incubation caused decrease of Fv/Fm to 0.4 (control 0.8); reduced Fm; increased NPQ; decreased qP; recovery during post-anoxia; anoxia stress was responsible for the reduction in PSII functionality.	Schlueter and Crawford 2003

3. 7. COMBINED WATERLOGGING AND SALINITY

The coincidence of anaerobiosis and salinity is rather common in both natural and agricultural ecosystems (Barrett-Lennard 2003; John *et al.* 1977). It is increasingly recognized that waterlogging together with salinity has substantial adverse effects on higher plants. Lack of aeration can exacerbate the problems of regulation of ion fluxes to the shoots in saline media (John *et al.* 1977). The increased salt concentrations result in increased transport to the root xylem and hence to the shoot, as hypoxia increases the permeability of root membranes to Na^+ and Cl^-. The combined stress of salinity and waterlogging is frequently encountered in the field but information on the concurrent effect in agricultural crops is still scant (Barrett-Lennard 2003; Barrett-Lennard *et al.* 1999; West and Taylor 1984).

Only a limited amount of studies on the simultaneous effect of oxygen deficiency and saline conditions on agricultural crops have been conducted (Ashraf 2003; Barrett-Lennard 2003; Barrett-Lennard *et al.* 1999; Huang *et al.* 1995b; Rogers and West 1993; West and Taylor 1984; see also Table 3. 4.). Waterlogging and salinity caused inhibition of photosynthetic capacity; the concurrent stress proved more inhibitory than the two stresses individually (Ashraf 2003). Increased soil salinity and waterlogging resulted in significant reduction in chlorophyll, soluble sugar content and yield in wheat (Gadallah 1999).

Na^+ and Cl^- concentrations at the sites of xylem loading in the roots are likely to be regulated to a large degree by H^+-gradients across the plasma membrane of the cortical cells. These H^+-gradients are maintained by H^+-ATPase activity and are expected to be impaired due to waterlogging induced ATP deficits (Barrett-Lennard 2003; Gibbs and Greenway 2003).

Competition exists between Na^+ and K^+ leading to a reduced level of internal K^+ at high external NaCl concentration. Waterlogging strongly inhibits active ion transport (Cheeseman and Hanson 1979) so that any outward transport of Na^+ is inhibited thus leading to high internal Na^+ concentration while K^+-influx

is simultaneously retarded. These conditions lead to passive influx of Na^+ with little K^+-influx, so that the K^+/Na^+ ratio in the shoot decreases many times under hypoxic conditions. Maintaining high cytoplasmic K^+, however, is essential to survive in saline waterlogged environments. Anaerobic treatment caused increased Na^+ and Cl^- concentrations in shoot tissue in barley and rice (John *et al.* 1977), wheat (Sharma and Swarup 1989), and rapeseed (Boem *et al.* 1996). Waterlogging inhibited the mechanism for Na^+ exclusion from roots (Drew 1988), allowing increased inflow of Na^+ with transpirational waterflow and decreased nutrient uptake (Barrett-Lennard 2003; Barrett-Lennard *et al.* 1999). Transport of Na^+ to the leaves is greatly enhanced, while active transport of K^+ is inhibited, so that the ratio Na^+/K^+ can increase by a factor of 100 and more. Such an abnormally large accumulation of Na^+ with simultaneously depression of K^+ is likely to cause interference with stomatal regulation (Armstrong and Drew 2002).

Ca^{2+} is important for the maintenance of K^+ transport in the presence of Na^+ (Shabala *et al.* 2003). K^+ uptake under anaerobic conditions can be improved by addition of Ca^{2+} to the external medium. A high Ca/Na ratio is important for maintaining membrane function, particularly enzyme activity (Greenway and Munns 1980).

Maintenance of cytosolic K^+/Na^+ ratio largely depends on the efficiency of transport systems located at the plasma and vacuolar membranes (Maathuis and Amtmann 1999; Shabala 2003). Therefore, the plant's ability to regulate root membrane transport activity in hypoxic conditions seems to be crucial for encoding their tolerance to combine salinity and waterlogging stress.

Table 3. 4.: Physiological stress responses to the combined effect of waterlogging and salinity

Species	Treatment	Major Findings	Reference
Cucurbita pepo	2 weeks after emergence plants were subjected to w/l and salinity (100 mol m^3 NaCl)	Numbers of adventitious roots increased with waterlogging by 45%; salinity alone did not affect number of adventitious roots; root biomass reduced by 35% in salinity, 42% in w/l, 77% in combined stress; fruit reduced by 42% in either w/l or salinity and 74% in combined stress.	Huang *et al.* 1995a
Eucalyptus grandis *E. globulus*	salt (75 and 150 mol m^{-3}) waterlogging and combined stress in 14 week old plants	Relative SDW reduction for salt (S), waterlogged (W) and SW treatments were 49, 77 and 80% for *E. grandis* and 28, 70 and 73% for *E.globulus*: combined stress led to bigger reduction than either treatment alone; Na^+ and Cl^- conc. increased significantly in W and S treatments, but more in S treatment; the combined stress SW caused largest increase; K^+ leaf conc. reduced most in S then SW then W treatment.	Marcar *et al.* 2002
Panicum antidotale	3 populations, 30 day old plants, 46 days aerated/stagnant solution 200 mol m^{-3} NaCl and combination of these	Combination of salinity and waterlogging proved to be more inhibitory to all populations than the separate treatments; Chl *a* and *b* decreased; net CO_2 assimilation (Pn) transpiration rate, stomatal conductance, relative intercellular CO_2 concentrations (Ci/Ca), water use efficiency (Pn/E) decreased significantly under all stress conditions; there were population differences.	Ashraf 2003
Six Trifolium species	~ 6 week old plants were treated with 0, and up to 60 mM NaCl and waterlogged for 5, 10, 15 d	Reduced SDW with increasing duration of flooding and with increasing external salinity; Combined stress of salinity and waterlogging was most inhibitory; species differed significantly in response to w/l and salinity; increasing shoot Na^+ and Cl^- with increasing NaCl conc., compounded when waterlogged; significant w/l x salinity interaction.	Rogers and West 1993
Triticum aestivum	22 d old plants in aerobic and anaerobic soil watered with NaCl	Both waterlogging and salinity synergise to increase Na^+, Ca^{2+} and Cl^- conc. in shoots; decreased stability of leaf membranes; K^+/Na^+ ratio in shoots decreased progressively with salinization; Chl *a* and *b* contents progressively decreased with increasing salinity; Kinetin application ameliorated the deleterious of salinity and oxygen deficiency.	Gadallah 1999
Vitis vinifera	cuttings, up to 90 mM NaCl waterlogged for 7 d	Biomass reduction in salinity; interaction between salinity and w/l caused a greater growth reduction; Na^+ conc increased in all tissues when dual stress was imposed; different cultivars responded significantly differently; waterlogging greatly increases the potential for salt toxicity.	West and Taylor 1984

Table 3. 4.: Physiological stress responses to the combined effect of waterlogging and salinity (continued)

Species	Treatment	Major Findings	Reference
*Summer squash (*Cucurbita pepo*)*	2 weeks after emergence plants were subjected to w/l and salinity (100 mol m^3 NaCl) for 14-21 d	Combination of waterlogging and salinity exacerbated the adverse effects of each factor alone, for photosynthetic rate (Pn), stomatal conductance (g_s), leaf chlorophyll and nitrogen, but not for leaf water potential. Plants which had only been waterlogged recovered gs, leaf water potential, chlorophyll content after seven days, plants subjected to both stresses did not recover these parameters in the same time.	Huang *et al.* 1995a
Cotton, wheat, sugarcane and rice	Salinity from <4 to >12 dS m^{-1} and watertable depth from<1 to 2-3 m	The combined impact of waterlogging and salinity was more harmful to crop yields when compared with effects of individulal stresses.	Kahlown and Azam 2002
Italian Alder	Four year old trees waterlogged with distilled water, 2% and 4.5% NaCl solution for 6-week period	66% of trees died if waterlogged in 2% NaCl solution, complete mortality occurred in trees waterlogged in 4.5% NaCl. Application of NaCl increased time to budburst	Percival *et al.* 1998
Phalaris aqutica L.	Gravel medium watered with 150 mol/m^3 NaCl and 10 mol/m^3 of $CaCl_2$ nutrient solution (quarter sea water strength)	Shoot growth reduced by an average of 58% compared with non-saline treatment. Field trials on waterlogged and saline sites seem to indicate that some accessions are more tolerant to the combined stress.	Oram *et al.* 2002
Eucalyptus camaldulensis *Dehn.*	*Eucalyptus camaldulensis* 7 months old clonal lines were waterlogged to 50% of their depth and salinity was incrementally increased to 300 mM NaCl over 17 d.	Some *Eucalyptus camaldulensis* clones performed better based on parameters such as total plant biomass, total leaf area, root dry weight under conditions of waterlogging and gradually increasing salinity than others. This indicates a wide potential variation in stress tolerance of trees from a single provenance.	Farrell *et al.* 1996

3. 8. POST-ANOXIC AND RECOVERY RESPONSES

3. 8. 1. General

Because no plant can survive indefinitely under anaerobic conditions an ability to deal with the effects of re-exposure to O_2 is important. Specifically, the metabolism of the reactive oxygen species generated during aerobic (Blokhina *et al.* 2003) metabolism is an important attribute of anoxia tolerance. One system involves superoxide dismutase (SOD), converting superoxide radicals to hydrogen peroxide, which is reduced to water by peroxidases or catalases (Drew 1997).

Plants, which return to aerobic conditions after a long-term period of hypoxia, have depleted carbohydrate reserves and many features of cellular metabolism might be impaired. Re-establishment of cellular function associated with growth occurs over some time after exposure to aerated conditions (Schlueter and Crawford 2003). Metabolic down-regulation is of great importance for long-term survival under anoxia. A balance needs to be achieved in the plant tissue between maintenance of essential functions and economical consumption of reserves, so that leaves have the capacity to repair on return to aerobic conditions (Setter and Waters 2003; Brändle and Crawford 1999). Aerobic gas exchange capacity recovered slowly in the leaf tissue of cranberry, reaching about 70% of the control value after 10 days post anoxia. Non-photochemical quenching mechanisms recovered rapidly under re-exposure to air. Photochemical quenching mechanisms on the other hand took longer to repair but seemed complete after a 10-day recovery period (Schlueter and Crawford 2003). For examples of recovery studies refer to Table 3.5.

3. 8. 2. Nutritional Aspects

Lizaso *et al.* (2001) found that flooded plants accumulated macronutrients quicker upon recovery than previously non-flooded plants, with P being the only exception. However, it usually takes a while for a plant's basic physiological functions to be recovered after a period of waterlogging. After short periods of oxygen deficiency and subsequent return to aerated conditions induced nutrient

stress was quickly reversed and electrical potential of cell membrane of beetroot was restored to pre-stress levels (Zhang *et al.* 1992). Resumption of oxygen supply resulted in K^+ uptake in cucumber roots (Bertoni *et al.* 1993).

3. 8. 3. Photosynthetic Characteristics

A number of researchers found that photosynthetic capacity of waterlogged plants recovered after a period of aerated conditions (see table 3. 4). Both gas exchange parameters (Ahmed *et al.* 2002), and pigment content (Huang *et al.* 1995b) were recovered. It was found that the capacity of recovery after hypoxic stress depended on factors such as length of stress, severity (Malik *et al.* 2002) and growth stage when waterlogging occured (Teutsch and Sulc 1997; Umaharan *et al.* 1997). Biomass is usually the slowest to recover (Davies *et al.* 2000b; Heinrichs 1970). Malik *et al.* (2002) found that seminal root growth in wheat did not recover even if the period of waterlogging only lasted 3 days. This was due to death of existing apices and no initiation of new lateral roots. As previously waterlogged plants have a smaller capacity for assimilation due to photosynthetic rates recovering slowly and fewer photosynthetically active leaves being available at the end of waterlogging it is understandable that full recovery of biomass cannot be expected in the short term.

Table 3. 5: Waterlogging and recovery

Species	Treatment	Major Findings	Reference
Annona species	Continuous flooding up to 50 d or flooding cycles with 2 or 4 weeks recovery	Growth rates were not regained after 4 weeks of suspending flooding (root system irreversibly impaired).	Núñez-Elisea *et al.* 1999
Cicer arietinum (chickpeas)	Waterlogged for 10 d then drained and recovered for 10 d	W/l tolerance decreased with increasing plant age, declining sharply at flowering; plants from which buds were removed showed the most rapid recovery; defoliation immediately before w/l reduced rate of recovery.	Cowie *et al.* 1996
Cucurbita pepo (squash)	Waterlogged and/or salinity treated for 14 d; recovery for 7 d	7 d after termination of w/l full recovery occurred for stomatal conductance, leaf water potential, leaf chlorophyll content and leaf and root N conc.; for w/l plants under saline conditions these parameters did not achieve that of control after recovery.	Huang *et al.* 1995b
Lupinus angustifolius, L. luteus	2 species of lupin w/l for 14 d at 28 or 56 DAS; drained and recovered for 14 d	Significant reduction in dry weight occurred following recovery; inadequate supply of photo-assimilates to the roots may have contributed to reduced root growth during recovery.	Davies *et al* 2000b
Medicago sativa	Flooded for 14 d at three different vegetative growth stages	The older, vegetatively advanced lucerne seedlings recovered shoot re-growth potentials after temporary flooding better than did seedlings in the early trifoliate stages; higher temperatures reduced recovery after flooding; plants began to show visible signs of recovery (greening up) 10 to 12 days after termination of flooding.	Teutsch and Sulc 1997
Pisum sativum	Anaerobiosis in pea leaves and chloroplasts was induced by flushing with N_2 gas, then returned to air	Anaerobiosis resulted in 10-20% reduction in the maximum quantum yield of primary photochemistry of PSII (Fv/Fm); time dependent recovery from anaerobiosis induced change; the Fv/Fm ratio recovered almost fully when leaves were returned to air.	Haldimann and Strasser 1999

Table 3. 5: Waterlogging and recovery (continued)

Species	Treatment	Major Findings	Reference
Triticum aestivum	13 or 20 d of hypoxia (N_2-bubbled nutrient solution); 60 mmol/m^3 NaCl; recovered for 13 days	Hypoxia increased net rate of Na^+ and Cl^- uptake in shoots; Na^+ and Cl^- conc. increased in the expanded but not in the expanding leaf; hypoxia caused 40-60 % decrease in growth rate; decreased net rate of K^+ uptake by 70-80 %, which resulted in 30-40 % decrease in K^+ in the youngest emerging leaf.	Barrett-Lennard *et al.* 1999
Triticum aestivum	w/l for 14 d at 100mm or 200mm below soil surface	Growth rates of plants w/l to 200mm of the soil surface recovered; those in the more severe w/l treatment only partially recovered; net photosynthesis also recovered to control values for the 200mm treatment but not for the 100mm treatment.	Malik *et al.* 2001
Triticum aestivum	w/l for various periods (3, 7, 14, 21, 28 d); recovery up to 25 d	Leaf N conc. in recovered plants of similar size was similar to controls; seminal root mass did not recover; non-structural carbohydrate (NSC) returned to control values in all plant tissues after recovery of 7 d.	Malik *et al.* 2002
Vigna radiata (mungbean)	2 cultivars; w/l for 8 d at the reproductive and vegetative growth stages and recovered for 8 d	Photosynthetic rate, transpiration rate and diffusive resistance all recovered in both cultivars to near normal values within 4 d of recovery in the reproductive and within 8 d in the vegetative stage; seed yield and biomass was reduced by 20 %.	Ahmed *et al.* 2002
Vigna unguiculata (cowpea)	4 cultivars of cowpea, cycles of 4 d hypoxia and 6 d recovery	All cultivars had the ability to recover completely and produce near to normal yields when w/l was confined to vegetative phase.	Umaharan *et al.* 1997

3. 9. WATERLOGGING AND LUCERNE

3. 9. 1. General

Agricultural development in south-eastern Australia as well as in the south west of Western Australia is characterized by the replacement of deep-rooted perennial native vegetation with shallow-rooted, winter annual crops and pastures. This causes excess rainfall to drain below the root zone and thus adds to ground water recharge. This development leads to shallow water tables, which are often saline due to mobilized salts. This leads ultimately to reduced plant growth and productivity (Dear *et al.* 2003; Latta *et al.* 2002).

Lucerne has been studied in view of its hydrological benefits in Australian cropping systems (Crawford and MacFarlane 1995; Latta *et al.* 2001; Latta *et al.* 2002; Lolicato 2000). Lucerne has been in cultivation for more than 2000 years and so long as lucerne is grown in well-drained soils almost any soil may be suitable for its production (McIntosh 1910).

Because of its high transpiration rate and due to its ability to go into a state of semi-dormancy in response to drought stress (Lolicato and Rogers 1997) it has suitable attributes for the management of waterlogged and/or saline sites. A rotation of 3 or more years of lucerne followed by several other crops such as wheat will increase the water use of south western Australian grain producing farming systems (Latta *et al.* 2001). This is a region were 1% of the land surface is lost annually to salinity and were rising water tables and salinity threaten 6 million hectares of land. Phase farming that includes a rotation of lucerne is a sound management option for farmers wishing to continue grain production in a sustainable way (Latta *et al.* 2002).

3. 9. 2. Physiological Limitations

Lucerne shows good tolerance to soil water deficit if an extensive root system can develop, on the other hand, waterlogging conditions are tolerated only if the periods of flooding are brief and excess water can drain away relatively quickly. Lucerne is not well adapted to prolonged periods of waterlogging and

shows no tolerance to the combined stress of salinity and waterlogging according to Evans (1998). Susceptibility of lucerne to waterlogged conditions is greater at higher temperature (Cameron 1973), but Cameron also pointed out that some lucerne cultivars are more tolerant of the stress than others. Flooding caused cessation of secondary growth and the suppression of lateral root growth and aerenchyma channels were formed in response to the stress (Zook *et al.* 1986). The formation of aerenchyma tissue in the stele of lucerne had not previously been reported. Zook *et al.* (1986) speculated that this mechanism could affect moderate flood tolerance by allowing translocation of oxygen to flooded roots. Heinrichs' (1970) study evaluated flooding tolerance of various legumes and determined lucerne to be tolerant to flooding for up to 15 days; however, this did depend on a number of external parameters, which can affect the severity of waterlogging injury. In a study reported in Kutschera (1960) lucerne was successfully established to pump excess water from a largely flooded riverside soil, with moving soil water. Here lucerne remained established for a period of 4 years and was not detrimentally affected by flooding, since the soil-water carried enough oxygen to allow lucerne to persist.

3. 9. 3. Conclusions

Plant adaptive responses to waterlogging are multiple and occur at various levels of plant structural organization. The switch to alcoholic fermentation and other biochemical changes is one manifestation of waterlogging tolerance. As these can't be easily measured by non-destructive techniques, identification of associated physiological responses to oxygen stress at the whole-plant level may lead to the identification and isolation of the genes conferring waterlogging tolerance. This is one possible avenue that will facilitate the selection and breeding of crops with greater resilience to hypoxia. Identification of genes regulating metabolic changes, tolerance or avoidance of oxygen deficiency may ultimately lead to modification of plant responses in an attempt at breeding more tolerant crops (Drew 1997). Also, considerations of energy metabolism alone cannot explain plant tolerance to flooding. Physiological changes that enable

anoxia to be avoided are essential, especially when faced with long-term flooding (Vartapetian and Jackson 1997). Physiological responses in lucerne to waterlogging indicate that individual plants show a greater level of tolerance (based on fluorescence characteristics) and this might lead to the identification of genetically superior material. If the variation of chlorophyll fluorescence correlates with waterlogging tolerance of individual plants then chlorophyll fluorescence might prove to be a useful selection tool for outstanding individuals, which could then be utilised in a breeding program.

CHAPTER 4

PHYSIOLOGICAL ASSESSMENT OF LUCERNE RESPONSES TO WATERLOGGING: EVALUATION OF POTENTIAL SCREENING METHODS[1]

4. 1. ABSTRACT

To facilitate the breeding process, efficient tools to screen a population of lucerne cultivars are needed. In this study, a comparative analysis of waterlogging effects on leaf photosynthesis, pigment composition, PS II photochemistry, and plant growth characteristics was undertaken using four different lucerne cultivars (Aurora, Hunter River, L153 and Sequel HR). Two-month-old plants, grown in half-strength Hoagland nutrient solution, were waterlogged for 16 days, and plant physiological characteristics were monitored at regular (every few days) intervals. All cultivars had significantly reduced fresh and dry weight for both shoots and roots after 16 days of waterlogging. The root biomass showed a greater percentage of reduction than did the shoot biomass. As waterlogging stress developed, chlorophyll content, CO_2 assimilation rate, transpiration rate, stomatal conductance and maximal quantum efficiency of PS II (Fv/Fm) decreased significantly. Chlorophyll *a* and *b* content gradually decreased over the time of the experiment in the stressed cultivars, and leaf chlorosis became increasingly evident. Although most of the parameters showed significant changes as waterlogging progressed, limitations, render some of them inapplicable for screening. It is concluded that for practical purposes of screening, the Fv/Fm ratio

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is the most appropriate. A significant difference between control and waterlogged plants became evident as early as at day seven. Possible physiological mechanisms involved are discussed.

4. 2. INTRODUCTION

Lucerne is being promoted to minimize groundwater recharge and lower water tables in cropping and grazing zones in temperate regions of Australia. Lucerne is susceptible to waterlogging stress and this is a serious constraint in areas with shallow water tables (Humphries and Auricht 2001). In these areas, unusually high winter rainfall over a short period can lead to waterlogged soils for an extended period. Even after very short periods of waterlogging lucerne plants are severely damaged (Evans 1998) meaning that large areas of lucerne can be lost after even a relatively short time of inundation.

For most plants, the capacity of roots to supply nutrients and water to the rest of the plant is inhibited in waterlogged soils (Moore and McFarlane 1998; De Simone *et al.* 2002). Low oxygen levels in the rhizosphere due to waterlogging reduces shoot and root growth (Kozlowski 1984). The detrimental effects of waterlogging on various crops have been demonstrated for a large number of species such as wheat (Huang *et al.* 1997), decurrent false aster, *Boltonia decurrens* (Smith and Moss 1998), and oak (Valentini *et al.* 1995; Wagner and Dreyer 1997). Waterlogging has been shown to affect some major physiological functions. A variety of plants demonstrated reduced stomatal conductance (Wagner and Dreyer 1997; Smith and Moss 1998) in response to root zone saturation. This reduction was often accompanied by reduced CO_2 assimilation (Wagner and Dreyer 1997; Valentini *et al.*1995), reduced growth (Musgrave and Ding 1998) and leaf chlorosis (Huang *et al.* 1994).

Genetic variation in response to waterlogging stress has been found in some plants. Huang *et al.* (1994) demonstrated genetic differences in tolerance to waterlogging in wheat cultivars. Genetic differences to waterlogging have been

reported for maize cultivars (Fausey *et al.* 1985) and different species of *Eucalyptus* (Van der Moetzel *et al.* 1988; Marcar *et al.* 2002).

Being an out-crossing species, lucerne plants show a high degree of genetic diversity (Musial *et al.* 2002) and variability in their physiological responses to environmental stresses. That means that many plants must be screened before conclusions are made. This also imposes a requirement for a method to be non-destructive, allowing the "outstanding" individuals to be later used in a breeding process. Obviously, not every physiological parameter or technique fits that description.

As photosynthetic machinery is very sensitive to all sorts of environmental perturbations, it is not surprising that chlorophyll fluorescence signals are widely used to assess the functioning of the PSII activity under various abiotic stresses such as heat, chilling, drought, waterlogging, and salinity (see Shabala 2002 for a review). Numerous papers published recommend the use of chlorophyll fluorescence as a most convenient screening tool (Mohammed *et al.* 1995).

Surprisingly, there are only a handful of reports on the use of the chlorophyll fluorescence technique to assess the photosynthetic performance in waterlogged plants. To my knowledge, no such attempt has been undertaken for lucerne plants. However, as prolonged waterlogging significantly impairs photosynthesis in many species, one would expect a similar type of response in lucerne genotypes. Two of the cultivars chosen for this study (Hunter River, Aurora) were or are still widely used in Australian cropping systems. For the other two genotypes some untested anecdotal evidence exists regarding their tolerance to waterlogging. Sequel HR is said to be waterlogging intolerant and L153 is reputedly tolerant to the stress.

The main objectives of this study were:

- To investigate the kinetics and the extent of waterlogging effects on chlorophyll fluorescence and other photosynthetic characteristics in lucerne species;

- To correlate the observed kinetics of chlorophyll fluorescence characteristics with associated changes in leaf pigment composition, CO_2 assimilation, transpiration and growth;

- To investigate whether there are differences in chlorophyll fluorescence between genotypes when exposed to waterlogging stress.

4. 3. MATERIALS AND METHODS

This project was carried out in a temperature-controlled glasshouse at the Horticultural Research Centre of the School of Agricultural Science at the University of Tasmania, in Hobart, Australia.

4. 3. 1. Plant Material and Growth Conditions

Four lucerne cultivars (Aurora, Hunter River, L153 and Sequel HR), supplied by SARDI (South Australian Research and Development Institute, Adelaide) were grown for about 60 days in steam sterilized 70:30 sand: perlite substrate in 1.8L pots, to which a ½ Hoagland nutrient solution was added every four hours by drip irrigation, controlled by solenoid valve and timer. The day/night temperatures were kept at 20°C ± 3°C under natural sunlight conditions. Seven replicates per cultivar and treatment were established. After emergence, lucerne was thinned to eight plants per pot. When plants were sufficiently robust to take measurements by an infra red gas analyser (IRGA) and pulse amplitude modulation PAM (~60 days after sowing), half of them were subjected to a waterlogged environment for 16 d. Waterlogged plants were placed on inverted pots in 10L buckets, which had an overflow outlet connected to a recirculating waterlogging system. Every four hours nutrient solution was pumped into the buckets and the overflow recirculated into the reservoir. Total dissolved oxygen concentration of the soil solution in the buckets was measured on day 15 of waterlogging in the recovery experiment (Chapter 5) using an oxygen meter (WTW OXI 330) at a depth of 30 mm in situ. Oxygen levels were rather small (around 2 mg L^{-1}) compared with air-saturated control measured (9.7 mg L^{-1}).

Aeration of the waterlogging solution was minimal, but possibly somewhat higher than under conditions in the field, since microbial respiration in soil can be expected to be higher than in a sterilized potting mix (see also Chapter 5, *Materials and Methods*). Pots were submerged in nutrient solution to a level as close as possible to the substrate surface line. To minimize evaporation and reduce algal growth in the nutrient solution a collar of sisallation sheeting (thick foil) was placed over each bucket, with a hole cut out the size of the pot area. New nutrient solution was added to the system as required.

4. 3. 2. Chlorophyll Content

From the beginning of the waterlogging treatment to just before the end of the experiment sequential extractions of chlorophyll (Chl) *a* and *b* were made (on days 1, 6, 11, and 16). A fresh weight (FW) collective sample of ~0.1g was taken, made up from 3-6 leaves using the third most recently fully expanded leaves of the plants sampled. Plant material was cut up into small (~3 mm^2) pieces and put in a vial. I added 10 mL of 96% methanol as well as a small amount (10 mg) of $MgCO_3$ to neutralize organic acids. The samples were immediately placed in the dark and kept at 4°C for 48 hours. A sub sample of 3.5 mL of the extract was taken and put in a spectrometer cuvette. The absorbance (optical density) was measured at wavelength 649 and 665 (OD_{649} and OD_{665} respectively) with a Perkin Elmer UV/VIS Spectrometer Lambda 20, (Bodenseewerk, Ueberlingen, Germany). Ratios of Chl *a* and *b* were calculated and compared with the stressed treatment. Seven replicates for each of the cultivars and treatments were analysed and Chl *a* and *b* content was averaged over the seven replicates.

The following formulae were used to calculate chlorophyll a and b content (Gusev 1982):

Chl *a* (mg/L) = 13.7 OD_{665}-5.76 OD_{649}

Chl *b* (mg/L) = 25.8 OD_{649}-7.6 OD_{665}

4. 3. 3. Biomass

Lucerne cultivars were subjected to waterlogged conditions for d 16 and then controls and stressed plants were harvested for biomass analysis. Plants were carefully taken out of the pot, while being immersed in water. Roots were gently washed under running water. Root loss during cleaning was kept to a minimum. Plants were patted dry with absorbent paper and roots were severed from the plant. Roots and shoots of each pot were bulked together and fresh weight of shoots and roots was measured with a Mettler BB2440 Delta Range balance (Mettler-Toledo, Greifensee, Switzerland). Plant material was dried at 65°C in a Unitherm Dryer (Birmingham, England) to constant weight and then the shoot and root dry weight was determined.

4. 3. 4. Gas Exchange

Gas exchange was measured with an LCI Portable Photosynthesis System IRGA (ADC BioScientific Ltd, Hoddesdon, England) on intact leaves between 12:00 and 14:00 hours on a clear day on days 2, 7, 11 and 15 of the experiment at ambient photosynthetic photon flux density. Photosynthetic rate (A), stomatal conductance (g_s), internal CO_2 concentration (C_i) and transpiration rate (E) were measured. The atmospheric pressure was 1000 mBar, ambient CO_2 concentration measured between 360-365 ppm. Measurements were taken on the third fully expanded leaf. The leaf, which was used to determine gas exchange, was severed and collected in a plastic bag and leaf area was measured soon after, using an ADC Area Meter AM100 (ADC BioScientific Ltd. Hoddesdon, England). The IRGA calculations were adjusted to the actual leaf area.

4. 3. 5. Chlorophyll Fluorescence

Chlorophyll fluorescence was measured at a temperature of 20 °C ± 3° C with a PAM portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) in conjunction with a leaf-clip holder 2030-B with integrated micro-quantum-sensor and temperature sensor (Walz GmbH, Effeltrich, Germany). All

measurements were carried out in the saturation pulse method described in the Mini-PAM manual.

Fluorescence measurements on dark-adapted plants were taken predawn sequentially at 48-hour intervals during the course of the experiment. Measurements were made on the third fully expanded leaf, taking care to avoid the mid-rib. Five measurements were taken per pot and four pots per cultivar and treatment were used as replicates, making n = 20. The Fo value was measured under low irradiance (0.15 μmol photons $m^{-2}s^{-1}$) modulated measuring beam and Fm (maximal fluorescence) was induced by a 800 ms pulse of intense saturating white light. The fibreoptics-to-sample distance and light intensity were chosen, such that the Fo value remained under 500. Variable to maximum fluorescence ratio was then calculated as Fv/Fm = (Fm-Fo)/Fm. Fluorescence parameters Fv' and Fm' at steady state were measured at midday when ambient light was fairly stable. Measuring light was turned on and actinic light was applied for 30 sec at an intensity of ca 250 μmol photons $m^{-2}s^{-1}$ (photosynthetically active radiation) and Fm' was determined by applying a saturation pulse.

4. 3. 6. Data Analysis

Each treatment had seven replicates, which were arranged in a completely randomised design. The factorial treatment arrangement consisted of four genotypes, and two treatments (control and waterlogging). Treatment effects were determined by analysis of variance with *t*-test at a probability level of P = 0.05.

4. 4. RESULTS

4. 4. 1. Growth Analysis

Prolonged (16 d) waterlogging caused significant changes in plant growth characteristics (Fig. 4.1; Table 4.1). With the only exception the Hunter River shoot DW, there was a significant decrease in both root and shoot biomass in

waterlogged plants (Table 4.1) in all studied cultivars. Most growth parameters of waterlogged plants were significantly different from those of the control plants.

The most severe effect was noticed in the root biomass, where root biomass of the stressed plants was only about 30% of controls (Fig. 4.1B). Although the impact of waterlogging on shoot growth was less severe (Fig. 4.1B; table 4.1), symptoms of waterlogging were visually quite obvious (Fig. 4.1A).

No significant differences in growth responses among the four cultivars studied were found after 16 d of waterlogging (Fig. 4.1B).

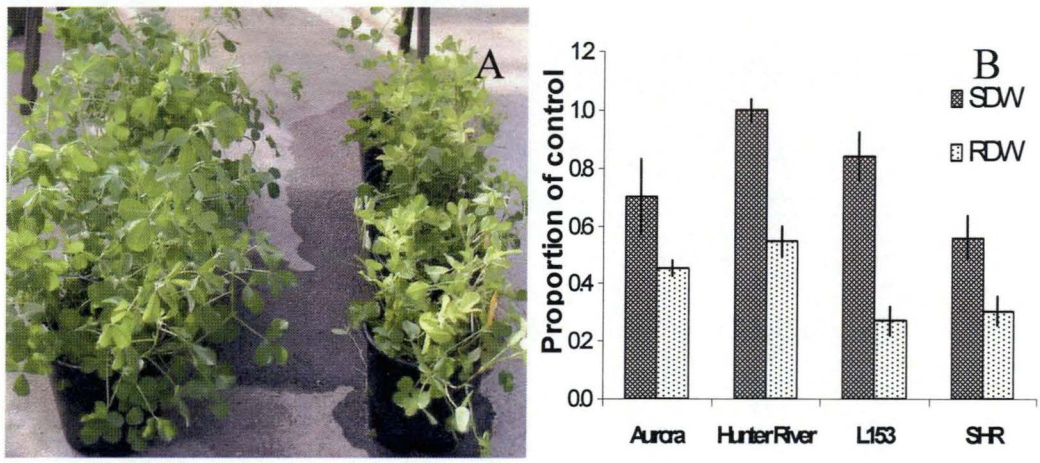

Figure 4. 1. Effect of waterlogging on plant growth characteristics. (A) - control and waterlogged Aurora plants 16 d after stress onset. (B) - changes in root and shoot DW for four different lucerne cultivars after 16 d of waterlogging. Normalized average values are shown. Data are mean ± s.e.m. (n = 4 - 7)

4. 4. 2. Chlorophyll Content

Leaf pigment composition was strongly affected by waterlogging. During the first few days after the onset of waterlogging, Chl *a* and *b* content in waterlogged plants increased slightly (Fig. 4.2^{A}). Then chlorophyll content started to decline, and the significant decrease in Chl *a* content between control and waterlogged plants became evident after day 11. By the end of the experiment, there was a 50% decrease in chlorophyll *a* content (Fig. 4.2^{A}A). A similar trend was obvious for total chlorophyll (Fig. 4.2^{A}C). Chl *b* values returned to their

original values (Fig. 4.2[A]B). The Chl *a/b* ratio declined more dramatically in waterlogged plants than in the control (Fig. 4.2[A]D). On the basis of the chlorophyll data, waterlogging had a significantly (P = 0.05) higher impact on cultivar L153 than on Sequel HR (Fig. 4.2[A]).

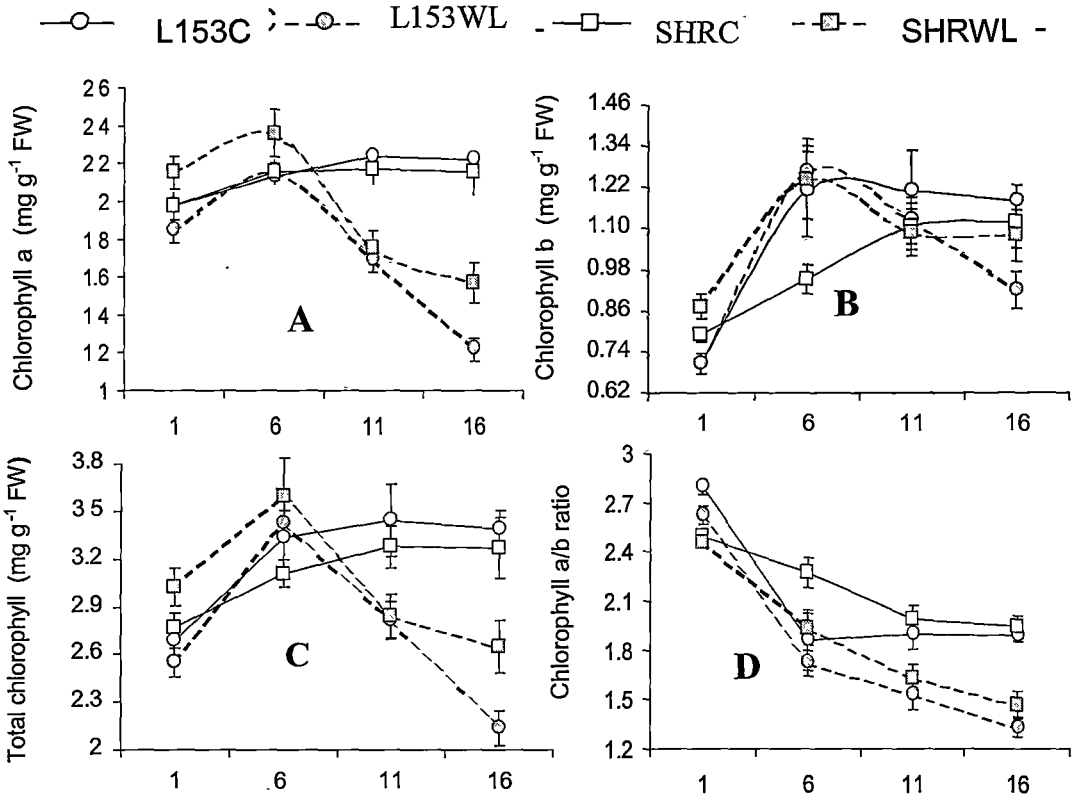

Figure 4. 2.[A] Effect of waterlogging on leaf pigment composition in two lucerne cultivars, L153 and Sequel HR. Data are mean ± s.e.m. (n = 7).

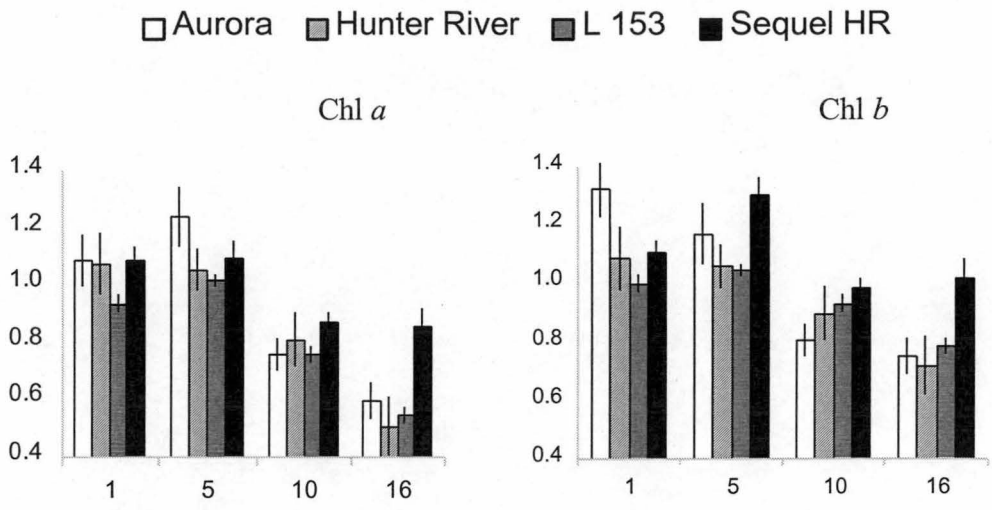

Figure 4.2.[B] Effect of waterlogging on normalized leaf pigment composition (chlorophyll *a* and chlorophyll *b*) in four lucerne cultivars, Aurora, Hunter River, L 153 and Sequel HR, over time. Data are mean ± s.e.m. (n = 7)

4. 4. 3. Gas Exchange Parameters

In all cultivars studied, waterlogging caused significant changes in leaf gas exchange characteristics (Figs 4.3, 4.4; Table 4.2). Net CO_2 assimilation in control plants was 25-35 µmol CO_2 $m^{-2}s^{-1}$ (Fig. 4.3A), while transpiration rate was around 12 mmol H_2O $m^{-2}s^{-1}$. The observed fluctuations in the absolute values of assimilation and transpiration rates may be explained by the variation in ambient light conditions on the day of the measurements. In waterlogged plants, photosynthetic rates started to decline 7 d after stress onset in all cultivars and were about half the rate observed in the controls on day 15 (Fig 4.3).

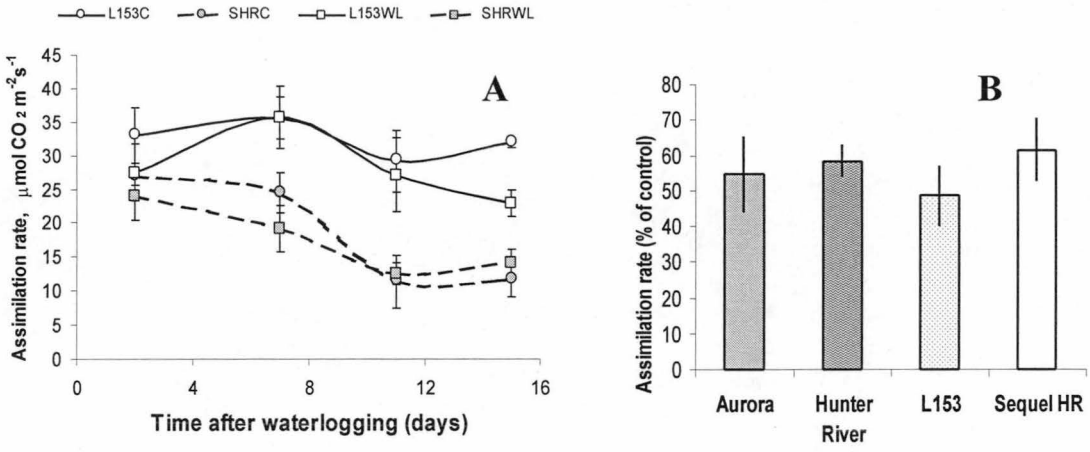

Figure 4. 3. Effect of waterlogging on net CO_2 assimilation by lucerne leaves. (A) Kinetics of net CO_2 assimilation over 16 d of waterlogging stress in L153 and Sequel HR plants. (B) overall changes in CO_2 assimilation for four lucerne cultivars (proportion to control) at day 15. Data are mean ± s.e.m. (n = 5).

The rate of transpiration in waterlogged plants was about 4.4 mmol H_2O $m^{-2}s^{-1}$ on average, which equates to about 30% of control values. Stomatal conductance for CO_2 (gs) also significantly decreased 7 d after waterlogging for all cultivars (data not shown) and remained low for the duration of the experiment, reaching values of 20 % of control for Hunter River, 10 % for Aurora, 15 % for L153 and about 17% for Sequel HR. Once again, no significant difference in effects of waterlogging on leaf gas exchange parameters among the four different cultivars studied was found (Figs 4.3B, 4.4B).

Intercellular CO_2 partial pressure reduced over time in waterlogged lucerne, noticeably on day 11 and 15 across all cultivars (see Table 4.2), which might partially be responsible for the reduced photosynthetic capacity.

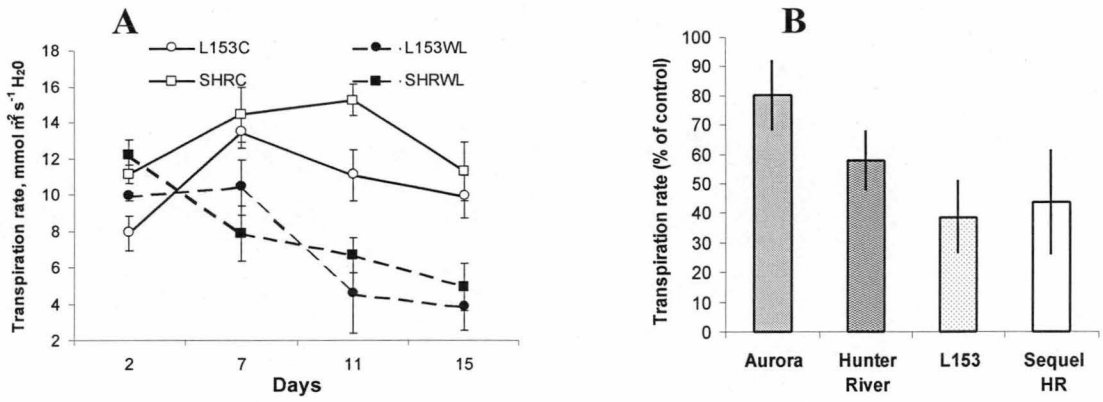

Figure 4. 4. Effect of waterlogging on rate of transpiration in lucerne leaves. (A) Changes in leaf transpiration rate over 16 d of waterlogging stress in L153 and Sequel HR plants. (B) Overall changes in the rate of transpiration for four lucerne cultivars (proportion to control) at day 15. Data are mean ± s.e.m. (n =5).

4. 4. 4. Chlorophyll Fluorescence

Most of the chlorophyll fluorescence characteristics were significantly affected by waterlogging (Table 4.3). Values of Fo and non-photochemical quenching (NPQ) significantly increased while values of Fm, Fm', Fv/Fm, yield (Y), and electron transport rate (ETR) significantly declined by day 15 (Table 4.3). Normalised values revealed no significant difference among the four cultivars used (Table 4.4). Absolute values of Fv/Fm showed an incremental reduction over time (see Fig 4.5).

Table 4. 1. Effect of waterlogging on biomass characteristics of four lucerne cultivars. Root and shoot fresh and dry weights were measured on day 16 after onset of waterlogging. Data are mean ± s.e.m. (n = 4 to 7 collective samples, 8 plants each). Significant (t-test) compared with control at:*, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$

Cultivar	Shoots		Roots	
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
Aurora				
Control	4.82 ± 0.23	0.66 ± 0.04	3.05 ± 0.30	0.25 ± 0.03
WL	2.23 ± 0.38**	0.46 ± 0.08*	1.01 ± 0.08***	0.11 ± 0.01*
Hunter River				
Control	3.67 ± 0.56	0.56 ± 0.07	2.84 ± 0.45	0.25 ± 0.04
WL	2.61 ± 0.10	0.56 ± 0.02	1.23 ± 0.14**	0.14 ± 0.02**
L153				
Control	5.54 ± 0.49	1.07 ± 0.10	5.50 ± 0.48	0.95 ± 0.13
WL	3.26 ± 0.32**	0.90 ± 0.09	1.58 ± 0.26***	0.26 ± 0.04***
Sequel HR				
Control	6.58 ± 0.61	1.16 ± 0.09	5.72 ± 0.28	0.87 ± 0.05
WL	2.35 ± 0.31***	0.65 ± 0.09**	1.80 ± 0.26***	0.27 ± 0.03***

Table 4.2 Average internal CO_2 concentrations (C_i) of four lucerne cultivars in control and waterlogged conditions. IRGA measurements were taken on day 2, 7, 11 and 15. Data are mean ± s.e.m. (n= 5).

Cultivar	Day	Control	Waterlogged	Difference
Aurora	2	236	230	6
	7	288	293	-5
	11	233	208	25
	15	245	202	43
Hunter River	2	233	237	-4
	7	294	294	0
	11	246	232	12
	15	246	212	34
L153	2	237	256	-19
	7	251	206	45
	11	248	160	88
	15	241	153	88
Sequel HR	2	261	267	-6
	7	221	180	41
	11	265	220	45
	15	243	177	66

Table 4. 3. Parameters of chlorophyll fluorescence after 15 d of waterlogging. Data are mean ± s.e.m. (n = 20). *, significant compared with control at $P = 0.05$ (t-test)

	Fo	Fm	Fv/Fm	Fo'	Fm'	Yield	ETR	NPQ
Aurora								
Control	364 ± 4	2033 ± 25	0.820 ± 0.002	301 ± 7	655 ± 31	0.528 ± 0.016	88.7 ± 6.3	2.11 ± 0.13
Waterlogged	447 ± 16*	1818 ± 41*	0.750 ± 0.012*	307 ± 13	590 ± 29	0.458 ± 0.032	50.4 ± 4.3*	2.36 ± 0.18
Hunter River								
Control	354 ± 4	2000 ± 32	0.822 ± 0.002	302 ± 10	671 ± 16	0.550 ± 0.011	74.5 ± 5.4	1.95 ± 0.07
Waterlogged	393 ± 8*	1799 ± 34*	0.780 ± 0.005*	272 ± 11*	507 ± 32*	0.436 ± 0.028*	63.0 ± 5.1	3.23 ± 0.03*
L 153								
Control	237 ± 5	1404 ± 25	0.830 ± 0.002	324 ± 7	850 ± 17	0.618 ± 0.007	69.2 ± 2.3	1.32 ± 0.05
Waterlogged	306 ± 12*	1288 ± 41*	0.756 ± 0.014*	330 ± 14	703 ± 46*	0.506 ± 0.024*	52.1 ± 2.6*	2.14 ± 0.31*
Sequel HR								
Control	230 ± 2	1362 ± 14	0.830 ± 0.001	322 ± 7	855 ± 27	0.621 ± 0.005	63.7 ± 1.1	1.34 ± 0.08
Waterlogged	285 ± 5*	1367 ± 27	0.790 ± 0.004*	349 ± 11	766 ± 38	0.529 ± 0.019*	54.6 ± 1.8*	1.72 ± 0.18*

Table 4. 4. Relative changes in chlorophyll fluorescence characteristics after 15 d of waterlogging. Ratios of corresponding values for waterlogged and control plants (given in Table 4. 3) are shown. Values >1 indicate an increase in a corresponding parameter; those <1 are indicative of decline.

Cultivar	Fo	Fm	Fv/Fm	Fo'	Fm'	Yield	ETR	NPQ
Aurora	1.22	0.89	0.91	1.01	0.9	0.87	0.57	1.12
Hunter River	1.11	0.90	0.95	0.9	0.76	0.79	0.84	1.65
L 153	1.29	0.92	0.91	1.02	0.83	0.82	0.75	1.62
Sequel HR	1.24	0.99	0.95	1.08	0.89	0.85	0.86	1.29

When plotted against time, the most obvious was the trend in the maximum quantum yield of PS II (Fv/Fm ratio; Fig. 4.5). Fv/Fm values steadily declined in the waterlogged plants over the course of the experiment. Fv/Fm of control plants remained constant at around 0.83 for all cultivars during the experiment, whereas Fv/Fm of the waterlogged lucerne plants decreased noticeably after day 7. The most affected were Aurora plants (Fv/Fm = 0.756), while Sequel HR appears to be the most tolerant to waterlogging (Fig. 4.5; Table 4.3). The difference in Fv/Fm values for these two cultivars was significant (P = 0.05) after day 13. Maximum fluorescence Fm remained relatively constant initially but decreased significantly at days 13 and 15 in the waterlogged plants (data not shown).

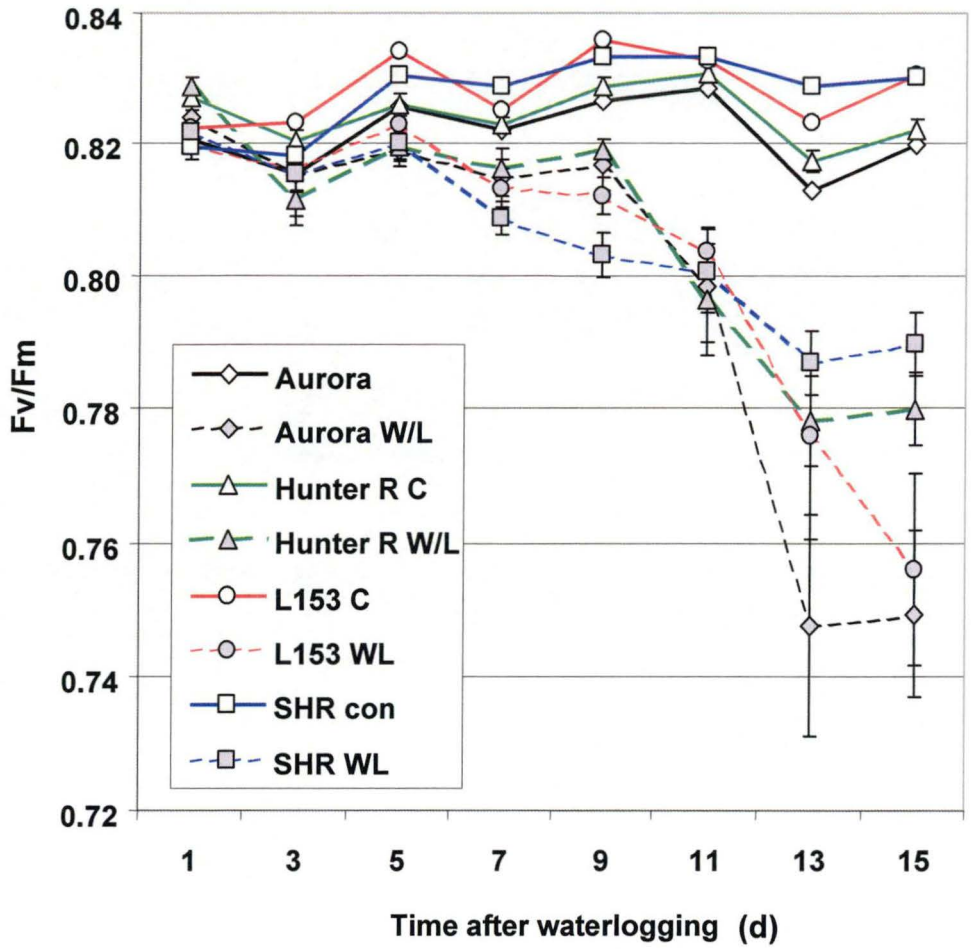

Figure 4. 5. Kinetics of Fv/Fm (maximal quantum efficiency of PSII) over 16 d of root waterlogging in four lucerne cultivars. Data are mean $\pm$ s.e.m. (n = 20).

4. 4. 5. Visual Assessment

Another typical symptom of waterlogging was the appearance of the red pigmentation on plant stems (Fig. 4.6A), presumably due to increased level of anthocyanin production. No significant difference was found among cultivars, when stem colouration was numerically scored (Fig. 4.6 B).

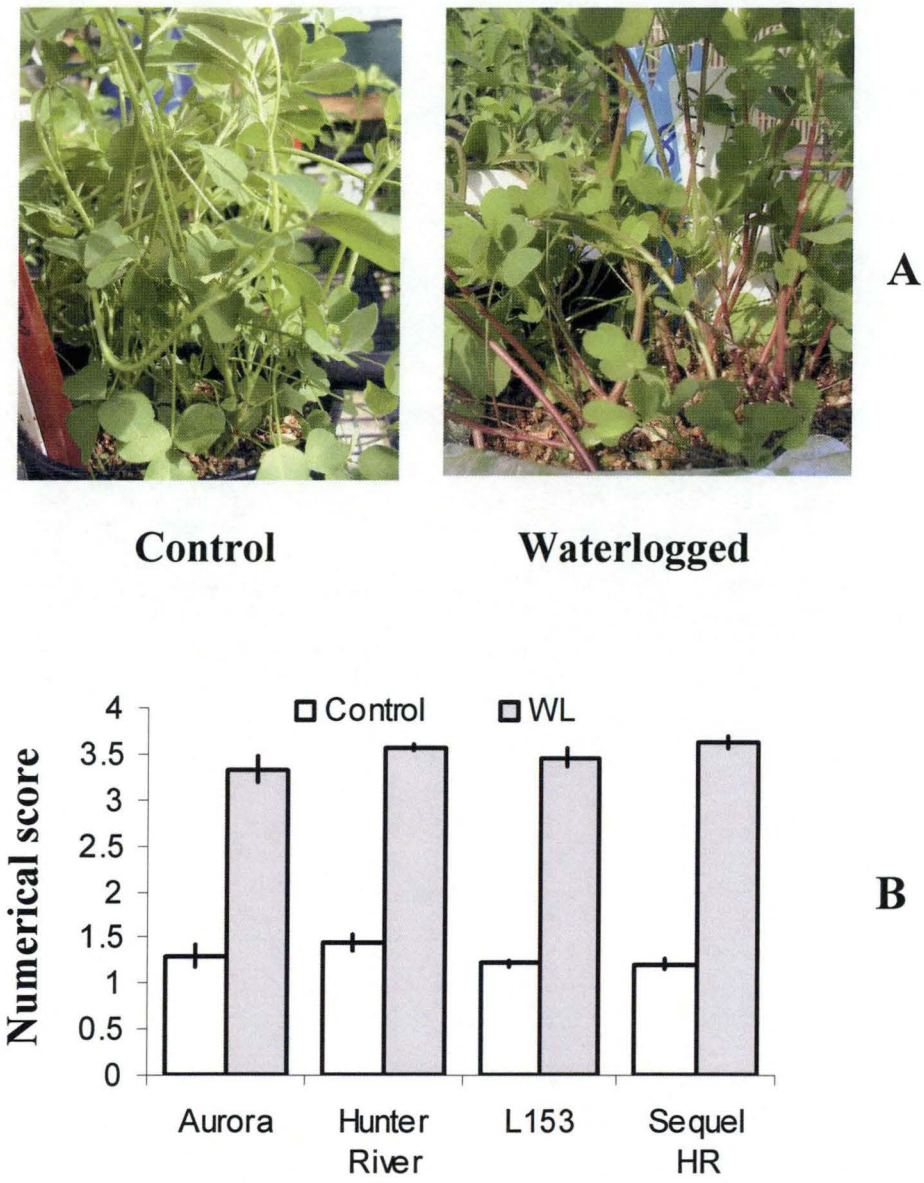

Figure 4. 6. Effect of waterlogging on stem pigmentation in waterlogged plants. (A) Control and waterlogged plants after 15 d of stress. (B) Numerical scoring of stem colour (1 to 4). 1, no red pigmentation; 2, light red; 3, moderately red; 4, intensively red. Data are mean ± s.e.m. (n = 7)

Levels of chlorosis of the three most recently fully expanded leaves were monitored and scored on the day of harvest (d 16). Chlorosis of all waterlogged cultivars was significantly higher than in the controls (see Fig. 4.6).

4. 5. DISCUSSION

4. 5. 1. Photosynthesis

With the exception of rice, most crop species are not adapted to waterlogging (Marschner 1995). Not surprising, therefore, that prolonged waterlogging may have a significant impact on plant photosynthesis (Vu and Yelenosky 1991; Takele and McDavid 1994; Davies *et al.* 2000b). In this study, net CO_2 assimilation (Fig. 4.3), leaf transpiration rate (Fig. 4.4) and stomatal conductance were all significantly reduced by waterlogging, with up to 60% reduction in net CO_2 assimilation rate after 15 d of stress application. However, no significant difference among lucerne cultivars was observed. This is consistent with reports by Musgrave (1994), who demonstrated that, in spite of severe limitations on photosynthesis caused by waterlogging, no significant differences among eight winter wheat cultivars were found.

4. 5. 2. Plant Growth

Reduction in photosynthetic performance was mirrored by greatly reduced biomass. Other investigators (Webb and Fletcher 1996; Musgrave and Ding 1998) found that sustained waterlogging reduced biomass in the plants under their investigation. Huang *et al.* (1997) compared two wheat cultivars differing in their tolerance to waterlogging and found that both cultivars were equally affected in biomass reduction. This is consistent with our data (Fig. 4.1; Table 4.1).

4. 5. 3. Chlorophyll Content

Chlorosis of leaves is often one of the early symptoms of stress and is associated with concomitant decline in concentration of photosynthetic pigment

(Webb and Fletcher 1996). Visual symptoms of waterlogging stress are the yellowing of the foliage and leaf wilting. These were obvious signs in this study (Fig. 4.1A). Daugherty and Musgrave (1994) found a substantial reduction (more than 50%) of total chlorophyll in *Brassica rapa* L. after 7 d of waterlogging. Huang *et al.* (1997) also demonstrated that waterlogging reduced wheat chlorophyll content by 20 and 40 % in waterlogging-tolerant and -sensitive species, respectively. Daugherty *et al.* (1994) showed a difference in response to waterlogging in two populations of *B. rapa* after 4 d of soil water inundation. The sensitive population had lower chlorophyll content than the more tolerant one. These results confirm my findings of increasing levels of chlorosis over the time of the experiment. After a slight initial increase in the Chl *a* level, there was a sharp decline in both Chl *a* and total chlorophyll content in leaves of waterlogged plants (Fig.4.2^{A}). By day 15 the Chl *a* content across all cultivars was reduced by 30-50% (Fig. 4.2^{A}A).

4. 5. 4. Chlorophyll Fluorescence

For close to twenty years chlorophyll fluorescence parameters have been advocated as non-destructive and non-invasive tools for early stress detection (Bolhar-Nordenkampf *et al.* 1989; Bjoerkman and Demmig-Adams 1994; Havaux 1992; Lichtenthaler 1988; Shabala 2002). As mentioned earlier, environmental stresses have been monitored and evaluated by chlorophyll fluorescence, however, chlorophyll fluorescence has not been widely used to closely scrutinize waterlogged plants.

Some investigators have found that the maximal quantum efficiency (Fv/Fm) has been reduced after imposing waterlogging stress on oaks (Wagner and Dreyer 1997), wheat (Webb and Fletcher 1996) and Italian elder, *Alnus cordata* (Percival *et al.* 1998). In this study, I showed that chlorophyll fluorescence characteristics of lucerne leaves are also significantly affected by root waterlogging.

Fv/Fm is a measure widely used to determine effects of environmental stresses on the photosynthetic apparatus and the resulting efficiency of the

quantum yield (Maxwell and Johnson 2000; Shabala 2002). Measuring the predawn value of Fv/Fm every second day throughout the experiment allowed close investigation of parameter changes over time and across treatments. The results consistently showed close approximation of Fv/Fm for controls at all times to the standard value of Fv/Fm values determined by Bjoerkman and Demmig (1987) of around 0.83. The Fv/Fm value for waterlogged lucerne, however, steadily declined after day 7 to as low as 0.75 on day 15 (Fig. 4.5; Table 4.3).

Other chlorophyll fluorescence characteristics were also affected by waterlogging stress. The lower Fm readings in the waterlogged plants (Table 4.4) seem to indicate a reduced efficiency of heat dissipation in these plants (Maxwell and Johnson 2000). The significant increase in Fo values (parameter of initial fluorescence of a dark-adapted sample) may indicate an impaired efficiency of coupling between antennae chlorophyll and reaction centres of PS II (Atwell *et al.* 1999). Increased minimal fluorescence (Fo) is often a sign of stress on the plant (Shabala 2002) and indicates that less energy reaches the reaction centres and therefore less energy is available for use in photochemistry.

4. 5. 5. Prospects for Screening

Although waterlogging affected all of the physiological characteristics studied, not all of these may be suitable for screening purposes. A sensitive indicator of waterlogging stress, chlorophyll analysis is a time consuming procedure. Gas exchange parameters are subject to fluctuations due to changes in ambient light intensity. Also, the use of the IRGA on lucerne is problematic owing to the smallness of lucerne leaves. Being an ultimate test for waterlogging tolerance, biomass analysis is a destructive method, which does not allow the "outstanding" individuals to be later used in a breeding program. That leaves chlorophyll fluorescence, and Fv/Fm measurements in particular, to be recommended as the most efficient tool to select individual plants tolerant to waterlogging. The average time required to measure Fv/Fm ratio from a pre-dark-adapted sample is only 2-3 seconds. That means that many hundreds of individual plants might be screened per day, providing scope for the discovery of individuals

that exhibit greater tolerance to the environmental stress of waterlogging. Another advantage is that for healthy (control) plants, the Fv/Fm ratio is remarkably constant in all cultivars and species (about 0.83, Maxwell and Johnson 2000; Table 4.4). Therefore, strictly speaking, there is even no need to measure this parameter from control plants. Field evaluations - following fluorescence screening in the glasshouse - are needed to verify the screening results.

4. 5. 6. Genetic Variability

Being an out-crossing species, lucerne shows a great deal of genetic variability. Not surprising therefore, that responses of individual lucerne plants to abiotic stresses, and particularly to waterlogging, may vary a lot (Rogers 1974).

This is further supported by our chlorophyll fluorescence data (Table 4.4; Fig. 4.5). The standard error for Fv/Fm measurements in control did not exceed 0.25% for all cultivars studied (Table 4.4). At the same time, in waterlogged plants, the standard error of the mean for Fv/Fm ranged from 0.5 to 1.8%. This may be an indication of a wide diversity of plant physiological responses to waterlogging in a studied population, within the same cultivar. It is obvious that some plants cope with stress better than others. This fact provides some promise for ultimately being able to select plants from a screening trial that consistently give a higher Fv/Fm reading than others from the same group, which can then be used to breed and multiply these promising lines.

CHAPTER 5

NUTRITIONAL AND CHLOROPHYLL FLUORESCENCE RESPONSES OF LUCERNE (*MEDICAGO SATIVA*) TO WATERLOGGING AND SUBSEQUENT RECOVERY[2]

5. 1. ABSTRACT

Periodic flooding of perennial crops such as lucerne (*Medicago sativa* L.) is a major cause of lowered productivity and leads in extreme cases to plant death. In this study, effects of waterlogging and subsequent recovery on plant nutrient composition and PSII photochemistry were studied to gain a better understanding of the mechanisms of recovery as they relate to leaf photochemistry (chlorophyll fluorescence) and nutrient dynamics. Three lucerne cultivars and one breeding line were flooded for 20 d, drained and left to recover for another 16 d under glasshouse conditions. Leaf and root nutrient composition (P, K, Ca, Mg, B, Cu and Zn) of waterlogged lucerne was significantly lower than in freely drained controls, leaf N concentrations were also significantly lower in waterlogged lucerne. At the same time, there were significantly (5-fold) higher concentrations of Fe in waterlogged roots and Na in leaves (2-fold) of stressed plants. PS II photochemistry, which was impaired due to waterlogging, recovered almost fully after 16 d of free drainage in all genotypes. Alongside fluorescence recovery, concentrations of several nutrients also increased in recovered plants. Growth parameters, however, remained suppressed after draining. The latter was due to both the smaller capacity of CO_2 assimilation in previously waterlogged plants (caused in part by nutrient deficiency and associated inhibition of PSII) and the

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[2] Published as: Smethurst C F, Garnett T and Shabala S 2004 Nutritional and chlorophyll fluorescence responses of lucerne (*Medicago sativa*) to waterlogging and subsequent recovery. **Plant and Soil** (in press)

plant's need to re-direct available nutrient and assimilate pools to repair the damage to the photosynthetic apparatus and roots. It is concluded, that for any lucerne-breeding program it is important to determine not only the degree of tolerance to waterlogging but also the potential for recovery of different genotypes, as well as look for "outstanding individuals" within each population.

5. 2. INTRODUCTION

So far there is no known waterlogging tolerant lucerne germplasm suitable for Australian conditions. There is waterlogging tolerant Russian germplasm, but this is characterised by extensive surface roots and no tap root and will not survive dry Australian summers (Rogers 1967). Although the four genotypes used in a previous study (Smethurst and Shabala 2003) were recommended by lucerne breeders as "likely to be contrasting", the difference between these was only marginal. Being a highly cross-pollinating species, most individual lucerne populations (i.e. cultivars) consist of a heterogeneous mixture of genetically heterozygous individuals. This fact seems to overshadow most of the differences between cultivars. It appears that for lucerne improvement, screening should be aimed at searching for outstanding (e.g. waterlogging tolerant) individuals rather than comparing between cultivars.

Finding tolerant germplasm through field trials is difficult, because the extent of waterlogging stress across trials can be extremely variable on a small scale. It is also difficult to rely on natural waterlogging events and applying waterlogging treatments artificially can be difficult. Last but not least, comparison of results from "natural" trails or field experiments is very difficult, if not impossible. This is especially important when the germplasm collection is screened by a large number of institutions (as in this project). For these reasons, I aim to develop a method to preliminarily screen large numbers of lucerne plants in glasshouse-based experiments so that more targeted germplasm can be evaluated in field trials. Smethurst and Shabala (2003) found that the Fv/Fm ratio (a measure of maximal quantum efficiency of PS II; Maxwell and Johnson 2000) showed promise as an indicator of tolerance, which can be easily applied to

individual plants. The Fv/Fm ratio progressively declined in all four studied cultivars after the onset of waterlogging stress. The reasons for this decline, however, remain unknown. One possibility is that changes in PSII photochemistry result from lowered Ci induced by stomatal closure (Lawlor and Cornic 2002). A reduction of ~50 μmol mol^{-1} from 240 to 190 μmol mol^{-1} was measured in a previous experiment after 15 days of waterloggging (Smethurst and Shabala; unpublished). However, while reduced stomatal conductance inevitably leads to reduced CO_2 assimilation (and thus to photosynthesis in general) and such chlorophyll fluorescence characteristics as ETR, it is not likely to affect maximal efficiency of PSII, Fv/Fm (Maxwell and Johnson 2000). Therefore it is important to separate the effect of waterlogging on stomatal and non-stomatal components of photosynthesis, as well as their subsequent recovery.

A possible reason for this response might be altered hormonal status (specifically, reduced cytokinins from roots; Salisbury and Ross 1992) or perturbations in mineral nutrient assimilation (Castonguay *et al.* 1993; Colin-Belgrand *et al.* 1991). However, to my knowledge, no information relating alterations in plant nutrient composition with chlorophyll fluorescence parameters in lucerne is available in the literature. Also, it is not clear to what extent photosynthetic machinery in waterlogged leaves may recover after waterlogging, and how such a recovery is associated with plant nutrient acquisition. Since pigment analysis and scoring of chlorosis (data not shown) clearly pointed to the fact of nutrient bleaching, reduced nutrient status is also a likely scenario for impaired PS II functionality. Uptake and translocation of nutrients within the plant are affected by oxygen deficient conditions and result in ion transport interference (Trought and Drew 1980b). Roots are the first to experience the oxygen depleted conditions in the root zone and often respond to waterlogging by reduced growth or even complete cessation of growth (Malik *et al.* 2002; Trought and Drew 1980a; Barrett Lennard *et al.* 1988; Drew 1983). Malik *et al.* (2002) found that total non-structural carbohydrates (TNC) had accumulated after three days of waterlogging in all plant parts of wheat. High levels of carbohydrate concentrations in leaf tissue may lead to feedback inhibition of photosynthesis and changes in the PSII photochemistry and thus influence photosynthetic capacity

(Godde 1999); thus multiple plant responses are generated in waterlogged conditions and changes in chlorophyll fluorescence are but one of many physiological reactions to waterlogging stress. The ease with which chlorophyll fluorescence characteristics are measured *in situ*, make it a potentially useful screening tool (Araus *et al.* 1998, Lichtenthaler 1988, Maxwell and Johnson 2000, and Mohammed *et al.* 1995). Though not a primary response, a decline in Fv/Fm is a good indicator of photoinhibitory impairment as a result of waterlogging stress, and, thus, might be used for the practical purpose of selecting "outstanding" individuals within heterozygous populations. None of the methods, dealing with "primary" plant responses to waterlogging (e.g. root characteristics) are appropriate for non-destructive large-scale (thousands of individual plants) screening.

The ideal waterlogging-tolerant cultivar would not just survive immediate waterlogging stress, but would also rapidly recover after periods of waterlogging. Recent studies suggest that mechanisms conferring plant resistance to waterlogging and subsequent recovery are strikingly different (Setter and Waters 2003). Therefore it was crucial to determine a set of physiological characteristics that might be used for rapid screening of resistant germplasm for each of these types of stresses. Chlorophyll fluorescence (and specifically the parameter Fv/Fm) is potentially a good candidate although to my knowledge no such work has been reported for lucerne. Here I investigated the recovery of plants after waterlogging ceased. In terms of reducing the impact of waterlogging on yield and also stand persistence, recovery from waterlogging could be as important as tolerance to waterlogging *per se*, which is particularly relevant for a perennial crop such as lucerne.

Accordingly, the specific aims of this study were:

- (1) to characterise the impact of waterlogging on nutrient composition of stressed and recovered lucerne,
- (2) to determine the extent of recovery of chlorophyll fluorescence and other physiological parameters after removal of waterlogging stress, and

- (3) to provide insight into genetic variability of plant responses to subsequent recovery from waterlogging in lucerne.

5. 3. MATERIALS AND METHODS

5. 3. 1. Plant Material and Growth Conditions

Three lucerne cultivars, (Aurora, Hunter River and Sequel HR) and one breeder's line (SARDI L153) supplied by SARDI (South Australian Research and Development Institute, Adelaide) were grown in steam-sterilized 70:30 sand:perlite substrate in 1.8L pots in a temperature-controlled glasshouse. The experiment was conducted in a greenhouse in early spring and into summer. The day/night temperatures were thermostatically controlled and were kept at 20°C ± 3° C and relative humidity was around 50%. The plants were grown under natural sunlight conditions. Light intensity varied throughout the experiment. Photon flux densities as low as 300 μmol $m^{-2}s^{-1}$ and as high as 1600 μmol $m^{-2}s^{-1}$ were recorded between 10:00 EST and 14:00 EST. Four replicates per cultivar and treatment were established. After emergence, plants were thinned to eight plants per pot, which constituted a single replicate. On d 1 of the waterlogging treatment height measurements of the main stems (n = 20) of each genotype and treatment were taken and baseline for height was established before waterlogging treatment started. There were no differences in height in either treatment at that time, reflecting little difference in biomass between treatments (data not shown). However, an initial harvest to determine biomass and nutrient concentrations, was not carried out. Treatment of control and waterlogged plants was essentially the same as in the previous experiment. Aeration within the pots themselves was negligible as the supply line was positioned at the edge of the bucket and the outlet well away from the pots. Total dissolved oxygen concentration of the soil solution in the buckets was measured on day 15 of waterlogging using an oxygen meter (WTW OXI 330) at a depth of 30 mm in situ. Oxygen levels were rather small (around 2 mg L^{-1}) compared with air-saturated control measured (9.7 mg L^{-1}). These values are likely to be higher than those experienced under field conditions, where soil microbial respiration is likely to exhaust oxygen almost

completely. However, it should be noted that some oxygen may have been introduced due to the sampling method employed.

Pots were submerged in nutrient solution to a level as close as possible to the soil surface. To minimize evaporation and reduce algal growth in the nutrient solution, a collar of siscillation sheeting (thick foil) was placed over each bucket, with a hole cut out the size of the pot area. Nutrient solution was replaced every 4 days in the 80L reservoir. At the end of waterlogging, one pot per genotype was harvested (8 plants) and the tissue analysed for nutrient content. There was little difference between the values for each genotype so an average value of all 4 genotypes was used. The remaining plants were recovered after the initial stress period of 20 days for 16 days to assess possible recovery of the fluorescence parameters and nutrient concentrations. At the end of the waterlogging period, pots were carefully taken out of the solution and allowed to drain freely on a wire-rack. Moisture content was monitored and watering using the same dripper system as used for the control plants, was resumed 24 hours after being taken out of waterlogged conditions. Control plants as well as recovering plants received half strength Hoagland's nutrient solution via drip irrigation six times per day, controlled by solenoid valve and timer. Plants were harvested and processed quickly and stored in a cool room as they were processed and then placed into the drying oven at 65°C at the end of the day. Roots were briskly agitated in four rinses of clean tapwater to dislodge adhering soil particles. Excess water was wicked away with paper towels and roots were processed quickly to minimize leaching of soluble plant nutrients (Misra 1994).

5. 3. 2. Plant Nutrient Analysis

Plant samples were dried at 65°C to constant weight. After biomass had been determined, stems, leaves and roots were separated and ground for further nutrient analysis. Tissue was digested according the method of Zarcinis *et al.* (1987). The method was modified by adding 1.5 mL of HCl, once the plant material had almost been fully digested with HNO_3 Tissues were analysed for P, K, Ca, Mg, S, B, Cu, Zn, Fe, Mn, and Na using an Inductively Coupled Plasma Optical Emission Spectrometer (ARL 3580 B). Lucerne hay standards were

analysed as reference material. Leaf tissue was ground to a powder and analysed for nitrogen using a Carlo Erba Nitrogen Analyser (NA 1500 Series 2 Total Combustion Gas Chromatograph).

5. 3. 3. Chlorophyll Fluorescence

Chlorophyll fluorescence was measured in vivo at a temperature of 20°C ± 3°C with a pulse-amplitude modulation portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). All measurements were carried out in the saturation pulse method described in the Mini-PAM manual. Fluorescence measurements on dark-adapted plants were taken after having been darkened for at least 30 minutes. Measurements were made on the third to fifth most recently fully expanded leaf. The same region was measured after recovery. Two measurements were taken per pot and four pots per cultivar and treatment were used as replicates (n = 8).

Determination of Fo (minimal fluorescence), Fm (maximal fluorescence) and Fv/Fm were essentially the same as described in the Materials and Methods section of chapter 4. Fluorescence parameters Fv' and Fm' at steady state were measured at midday keeping ambient light at ~200 µmol quanta $m^{-2}s^{-1}$. Measuring light was turned on and actinic light was applied for 30 sec. Fm' was determined by applying a saturation pulse of 10 000µmol quanta $m^{-2}s^{-1}$.

Quenching coefficients were calculated using the following equations:

- photochemical quenching, qP = (Fm'-Fo')/(Fm'-Fo)
- non-photochemical quenching, qN = (Fm-Fm')/(Fm-Fo)
- non-photochemical quenching, NPQ = (Fm-Fm')/Fm'

5. 3. 4. Data Analysis

Each treatment had three or four replicates, which were arranged in a completely randomized design. The factorial arrangement consisted of four genotypes and three treatments (control, waterlogging and recovery). Treatment effects were determined by analysis of variance and *t*-tests, and p = 0.05 used to indicate significance. Nutrient data was analysed using ANOVA to test for

statistical significance. Means were separated using least significant difference (LSD).

5. 4. RESULTS

5. 4. 1. Effect of Waterlogging on Plant Nutrient Status

There was a significant ($P = 0.05$) difference in the concentration of most analysed nutrients of various plant organs between waterlogged and control plants (Table 5.1). The only noticeable exception was sulphur, whose level remained unchanged in leaves. Also, P and Mg contents in lucerne stems of waterlogged plants were not significantly different from that in control plants. The greatest difference in macronutrient content was observed in leaves ($40 \pm 3.6\%$ on average) followed by stems (33 ± 2.2 %) followed by roots ($28 \pm 1.4\%$). Among micronutrients, there was a significant (about two-fold) difference in B, Cu and Zn content in leaves and stems (but not in roots) and a dramatic (almost five fold) variation in Fe concentration in roots. Also, a two to three-fold variation in Na content in plant aerial organs was observed (Table 5.1). After waterlogging all cultivars had increased levels of anthocyanin (red stem pigmentation) as well as severe leaf chlorosis (data not shown).

Root anatomy was also affected by waterlogging: we observed taproot disintegration and decay and lateral root formation, thick mats of adventitious roots close to the substrate surface, presumably to access areas of higher oxygen levels.

5. 4. 2. Growth and Chlorophyll Fluorescence Responses during Recovery

After 16 days of recovery from stress, visual symptoms of stress (such as leaf chlorosis and red stem pigmentation due to anthocyanin production) were absent and leaf tissue returned to its pre-stress appearance. It is important to stress that the "healthy" shoot appearance was not due to newly grown leaves, but occurred mainly as a result of re-pigmentation of (previously chlorotic) leaves. Photosynthetic machinery in lucerne leaves had nearly fully recovered as judged by chlorophyll fluorescence parameters. This is further illustrated in Figures 5.1-5.2 for four different cultivars.

Table 5. 1. Effect of waterlogging on nutrient composition in leaves, stems, roots at day 20 of waterlogging stress. Values represent means ± standard errors (n = 4 collective samples, 8 plants each); one pot per genotype sampled.

	Leaves		Stems		Roots	
Nutrients	Control	WL	Control	WL	Control	WL
	Macronutrients (% DW)					
N	5.48 ± 0.17	2.33 ± 0.34^{c}				
P	0.43 ± 0.05	0.23 ± 0.03^{a}	0.31 ± 0.05	0.24 ± 0.04	0.30 ± 0.01	0.25 ± 0.04
K	3.40 ± 0.20	2.16 ± 0.12^{b}	2.88 ± 0.28	1.83 ± 0.37^{a}	1.49 ± 0.16	0.96 ± 0.06
Ca	2.77 ± 0.13	1.46 ± 0.10^{b}	1.02 ± 0.09	0.63 ± 0.02^{b}	0.53 ± 0.06	0.42 ± 0.04
Mg	0.34 ± 0.08	0.23 ± 0.01^{a}	0.17 ± 0.01	0.23 ± 0.03	0.28 ± 0.03	0.19 ± 0.04
S	0.43 ± 0.02	0.42 ± 0.08	0.14 ± 0.01	0.17 ± 0.01^{a}	0.22 ± 0.02	0.15 ± 0.03
	Micronutrients (mg/kg DW)					
B	123 ± 9	61 ± 11^{b}	30 ± 2.7	16 ± 0.58^{b}	13 ± 1.1	11.5 ± 1.9
Cu	5 ± 0.52	2.26 ± 0.33^{b}	4.45 ± 0.47	2.03 ± 0.56^{b}	5.09 ± 0.22	7.01 ± 0.45^{b}
Zn	39 ± 3.8	18 ± 2.5^{b}	33 ± 4.7	18 ± 4.0^{a}	43 ± 4.2	47 ± 6.4
Fe	94 ± 8.9	74 ± 16.7	39 ± 13.8	44 ± 10.4	121 ± 13.0	585 ± 64^{c}
Na	211 ± 30	435 ± 142	154 ± 21	497 ± 33^{c}	617± 80	332 ± 93^{a}

Significant compared with control at $^{a}P = 0.05$, $^{b}P = 0.01$ and $^{c}P = 0.001$

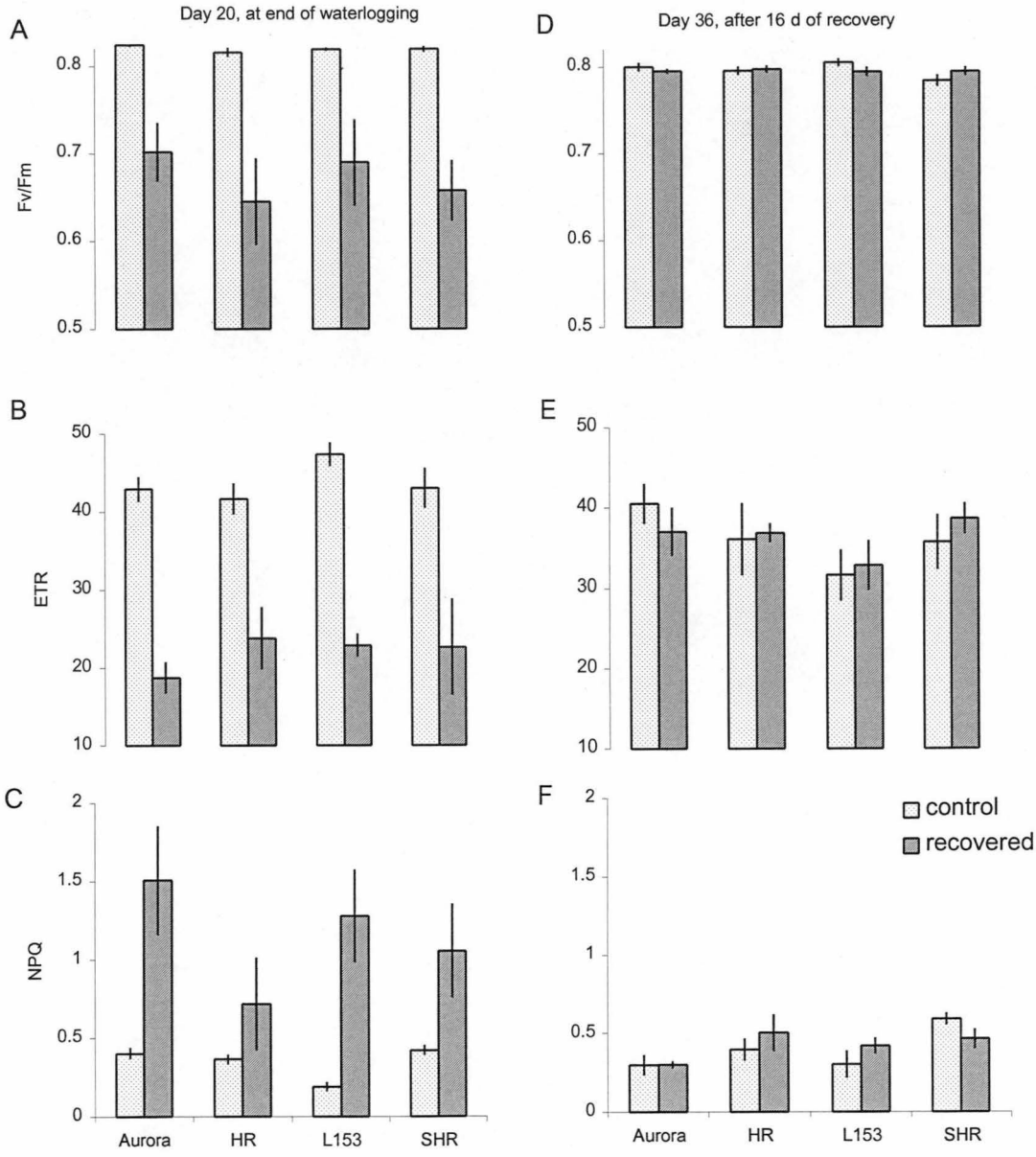

Figure 5. 1. Fluorescence parameters of lucerne after 20 d of waterlogging followed by 16 d recovery: maximum quantum yield of PSII (Fv/Fm) (A, D); electron transport rate (ETR) (B, E); non-photochemical quenching (NPQ) (C, F); error bars = SE, n = 8.

Similar to results in Chapter 4, a significant decline in Fv/Fm (the maximal efficiency of PS II photochemistry), from 0.82 to 0.67; $P = 0.05$, was observed after 20 days of waterlogging. By the end of the recovery period (16 days), this value returned back to around 0.8 (Figure 5.1; panel D). Quenching analysis also revealed a significant increase in qN and decrease in qP at the end of 20 d of waterlogging, and a near complete recovery in both these characteristics after 16 d of stress withdrawal (Figure 5.2; panels A-D). The apparent electron transport rate ($ETR = \Phi_{PSII}Yield \times PAR \times 0.5 \times 0.84$) after 20 days of waterlogging of

the stressed plants was only half of the controls (Figure 5.1; panel B) and showed near complete recovery by the end of the experiment (Figure 5.1; panel E).

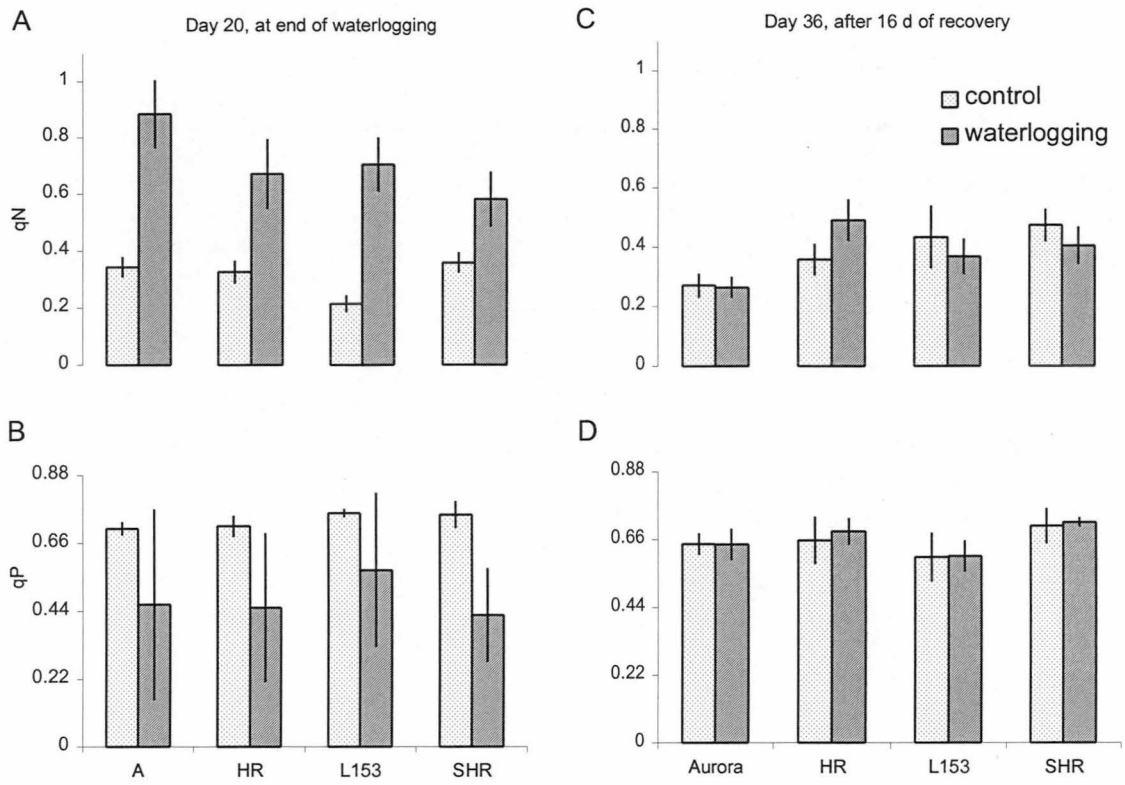

Figure 5. 2. Quenching parameters after 20 d of waterlogging followed by 16 d recovery: non-photochemical quenching, qN (Fm-Fm')/(Fm-Fo) (A, C); photochemical quenching qP, (Fm'-Fo')/(Fm'-Fo) (B, D); error bars = SE, n = 8

5. 4. 3. Nutritional Responses to Recovery

After 16 d recovery from waterlogging, nutrient concentrations of plant tissue in previously stressed plants changed back to near control levels (plants of the same age not subjected to waterlogging) at least in leaves. Tables 5.2-5.4 show absolute values of nutrient concentrations in leaves, stems and roots of the four cultivars at the end of recovery. In leaves, Fe and Na levels were significantly higher in recovered plants, while most other nutrients did not vary significantly (Table 5.2). There was a significant interaction for N; for the HR cultivar, concentrations in the waterlogged treatment were lower than those in the control, but waterlogging did not significantly affect the

Table 5. 2. Concentrations of nutrients in leaves of four cultivars in control plants (C) and plants recovered for 16 days after waterlogging (WL); significances of main effects (T = treatment, C = cultivar) and interactions (I) are indicated; (n = 3-4).

Nutrient	Aurora		HR		L153		SHR		Significant effects
	C	WL	C	WL	C	WL	C	WL	
Macronutrients (% DW)									
N	4.29	4.19	4.20	3.45	4.60	4.37	3.83	4.33	I, P = 0.02
P	0.31	0.32	0.29	0.34	0.32	0.35	0.29	0.34	ns
K	3.20	2.70	2.80	2.83	3.00	2.77	3.13	3.06	ns
Ca	2.37	2.40	2.57	2.47	2.60	2.10	2.56	2.41	ns
Mg	0.35	0.29	0.33	0.34	0.35	0.33	0.35	0.36	ns
S	0.42	0.42	0.38	0.44	0.38	0.42	0.43	0.39	ns
Micronutrients (mg/kg DW)									
B	104.7	94.9	109.8	97.4	139.37	89.35	101.46	99.47	T, P = 0.04
Cu	4.9	3.4	3.59	4.36	4.36	4.03	4.04	3.61	ns
Zn	30.9	26.4	27.1	36.9	30.08	35.37	27.47	33.49	ns
Fe	85.6	85.8	83.6	119.6	90.07	174.86	176.31	277.69	C, P = 0.02
Na	226.7	260.9	262.5	288.9	188.32	323.33	556.67	373	C, P = 0.04
Mn	97.2	61.8	83.35	61.37	77.86	56.87	76.54	62.01	T, P = 0.001

Table 5. 3. Concentrations of nutrients in stems of four cultivars in control plants (C) and plants recovered for 16 days after waterlogging (WL); significances of main effects (T = treatment, C = cultivar) and interactions (I) are indicated; (n = 3-4).

Nutrient	Aurora		HR		L153		SHR		Significant effects
	C	WL	C	WL	C	WL	C	WL	
Macronutrients (% DW)									
P	0.26	0.16	0.21	0.17	0.20	0.17	0.27	0.17	T, P = 0.01
K	2.73	1.90	2.56	2.53	2.46	2.40	2.90	2.73	ns
Ca	0.78	0.82	1.14	1.05	1.10	0.98	0.89	1.03	ns
Mg	0.19	0.19	0.21	0.24	0.21	0.27	0.20	0.22	ns
S	0.15	0.15	0.14	0.20	0.14	0.20	0.16	0.18	T, P = 0.02
Micronutrients (mg/kg DW)									
B	27.46	24.02	39.29	29.30	39.11	28.07	25.16	28.17	ns
Cu	3.93	2.47	3.13	3.37	3.84	3.09	3.51	3.47	T, P = 0.02
Zn	27.66	19.19	21.38	25.51	22.09	24.06	24.05	22.28	ns
Fe	29.55	27.86	26.61	42.85	38.24	72.73	37.50	66.57	ns
Na	250.00	336.66	185.33	356.66	200.75	480.00	300.00	333.33	T, P = 0.001
Mn	22.29	15.38	23.97	18.52	25.88	17.01	21.45	15.63	T, P = 0.001

Table 5. 4. Concentrations of nutrients in roots of four cultivars in control plants (C) and plants recovered for 16 days after waterlogging (WL); significances of main effects (T = treatment, C = cultivar) and interactions (I) are indicated; (n = 3-4).

Nutrient	Aurora		HR		L153		SHR		Significant effects
	C	WL	C	WL	C	WL	C	WL	
Macronutrients (% DW)									
P	0.25	0.26	0.18	0.16	0.18	0.17	0.22	0.12	ns
K	0.87	1.27	0.74	1.51	0.82	1.42	0.86	1.01	T, P = 0.001
Ca	0.48	0.68	0.44	0.52	0.62	0.55	0.49	0.42	ns
Mg	0.20	0.36	0.20	0.41	0.21	0.28	0.23	0.33	T, P = 0.001
S	0.17	0.25	0.14	0.26	0.16	0.22	0.16	0.20	T, P = 0.001
Micronutrients (mg/kg DW)									
B	10.84	15.94	10.66	14.34	11.32	14.50	11.01	10.71	T, P = 0.003
Cu	6.19	12.02	3.90	7.54	4.90	7.98	6.19	6.57	T, P = 0.008
Zn	34.76	68.77	19.89	39.48	22.90	34.82	36.16	30.01	ns
Fe	199.30	553.33	171.28	780.00	157.83	580.00	261.57	546.67	T, P = 0.001
Na	410.00	766.67	366.67	826.67	353.33	533.33	363.33	610.00	T, P = 0.001
Mn	54.86	42.29	29.37	43.32	35.41	41.87	76.46	47.70	ns

other genotypes (LSD = 0.749). Nitrogen concentrations in recovered lucerne leaves were similar to those of the control plants at the same age (Table 5.2).

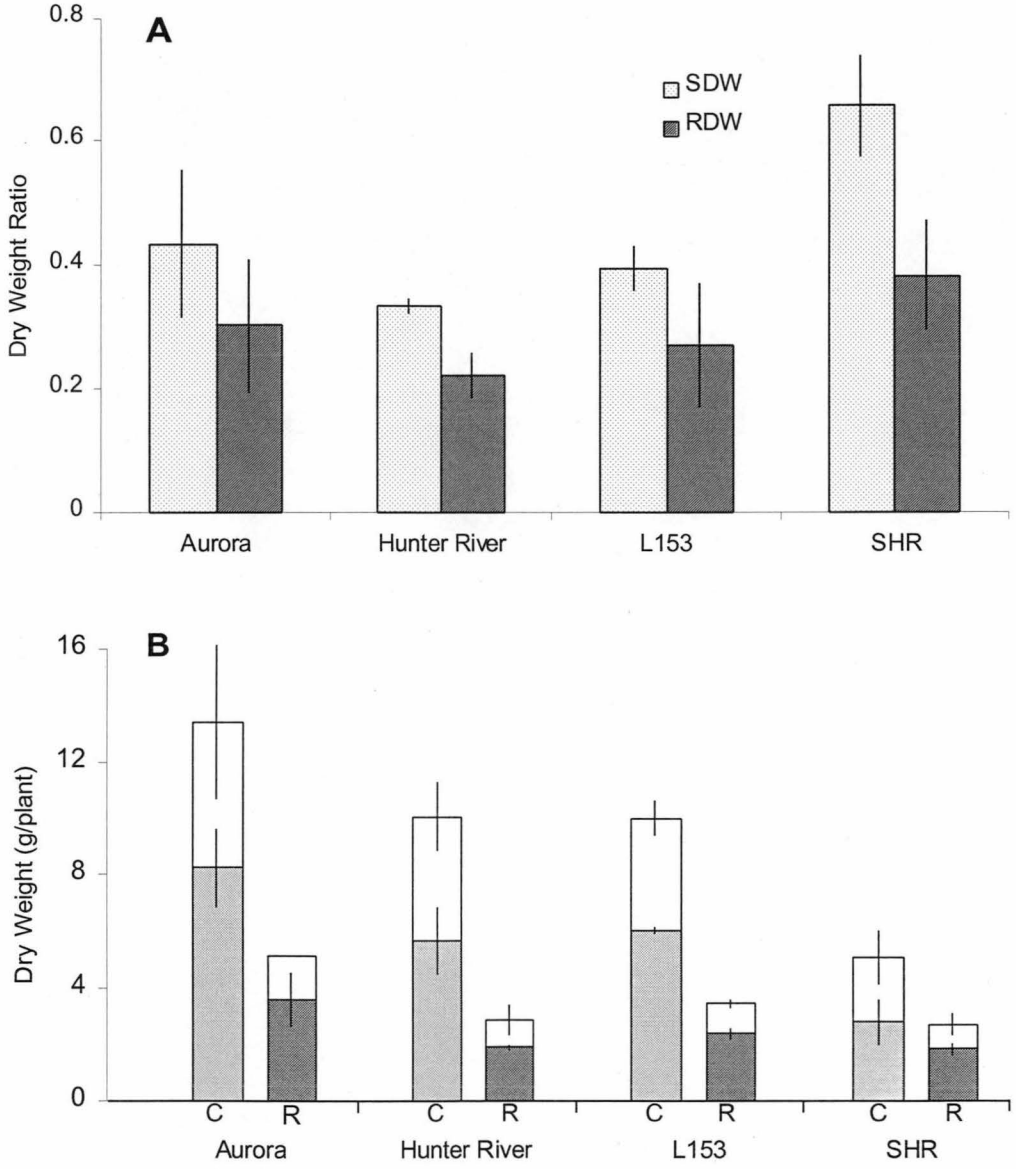

Figure 5. 3. Normalized biomass relative to control of shoots (SDW) and roots (RDW) of each cultivar after 16 d recovery (A) and total biomass at recovery (B); C denotes control plants, R denotes recovered plants, light area of the bars represents root biomass, the shaded area represents shoot biomass; error bars = SE, n = 3

In stems, recovered plants had significantly higher concentrations of Na, also significantly higher concentrations of S in most cultivars, and reduced

(compared to control plants) concentrations of P, Cu, and Mn. All other nutrients did not vary significantly.

In roots all genotypes had largely increased concentrations of most nutrients (Table 5.4), but not all differences were significant. The largest difference was the concentration of Fe (up to 300% above control) followed by the other nutrients. The only exceptions were phosphorous and calcium, whose concentrations in recovered roots were not significantly different to control plants for all cultivars. With Mn there was no clear response. Zn concentrations in roots were also not significantly different.

5. 4. 4. Genotypic Variability between Cultivars

The normalized values for biomass parameters after recovery were not significantly different between most cultivars. The only cultivar, which showed a less severe response to waterlogging was Sequel HR, which had a normalized RDW of 38% and a SDW of 66% in recovered plants (Figure 5.3; panel A).

However, absolute biomass of SHR was significantly lower than in the other cultivars (Figure 5.3; panel B). I observed considerable variation of all fluorescence parameters of stressed and recovered lucerne (Figures 5.1 - 5.2), although no clear cultivar response was discernable. At the same time, there was a rather wide range of variability in chlorophyll fluorescence characteristics measured in individual plants. This is further illustrated in Figure 5.4, where the range of Fv/Fm values are plotted. It appears that within the species, some plants showed almost perfect leaf photochemistry (as judged by Fv/Fm values, which should be close to 0.82 for every "healthy" plant; Maxwell and Johnson 2000), while most others showed a great degree of reduction in chlorophyll fluorescence characteristics.

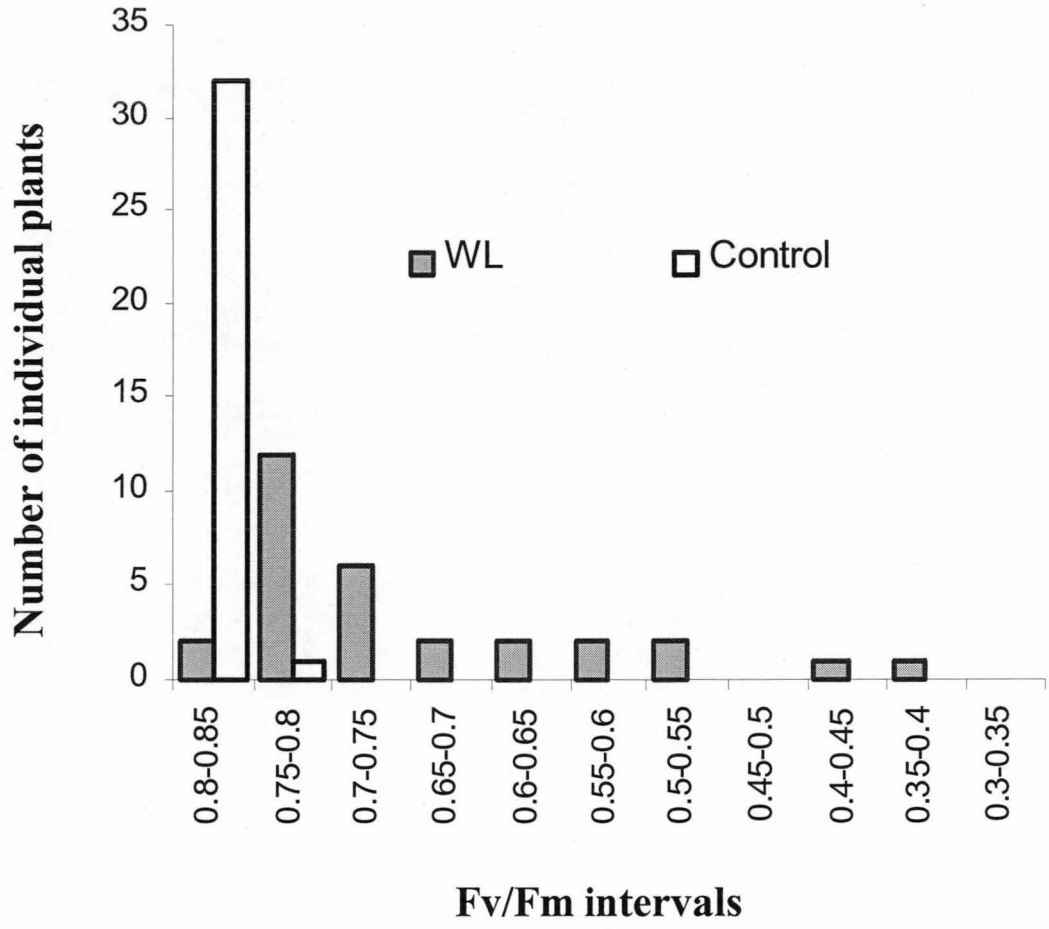

Figure 5. 4. Range of Fv/Fm values across all cultivars after 20 d of waterlogging of control (clear bars) and waterlogged plants (shaded bars).

5. 5. DISCUSSION

In waterlogged soils, air is displaced from the pore spaces, and oxygen is rapidly depleted, which changes the root chemical environment and affects root growth and nutrient acquisition (Gibbs and Greenway 2003). Most plants, including lucerne, are poorly adapted to waterlogging and develop leaf injury symptoms, such as wilting, chlorosis and epinasty (Drew 1990), which were also observed here. A decrease in hydraulic conductivity of the roots and accumulation of ethylene in the shoots are believed to be the main factors responsible for these symptoms.

Since lucerne is grown as a long-lived perennial crop, reduced productivity for a short time is an acceptable price to pay so long as the crop is able to persist and survive. Only a handful of papers have dealt with plant recovery after waterlogging (Davies *et al.* 2000a; Lizaso *et al.* 2001; Malik *et al.* 2001; Malik *et al.* 2002; Setter and Waters 2003; Barrett-Lennard 1999), and none of them describe chlorophyll fluorescence recovery kinetics for lucerne.

5. 5. 1. Biomass Recovery

Malik *et al.* (2002) found that wheat shoot biomass remained two to threefold lower in waterlogged treatments upon recovery compared with continuously drained controls. The extent of recovery was strongly dependent on the level of the watertable during flooding (Malik *et al.* 2001). The more severely waterlogged plants were only able to partially recover after 14 d of free drainage. Adaptation to waterlogging in lupins was studied by (Davies *et al.* 2000b), who found that growth response to hypoxia was more pronounced two weeks after waterlogging ceased than directly at the end of waterlogging. This result is confirmed in my study, where relative biomass production in stressed lucerne compared to controls (initial biomass before waterlogging commenced was not measured) was more reduced at the time of recovery than at the end of hypoxia (Figure 5.3). Baseline values of biomass and nutrient concentrations at the beginning of the waterlogging treatment would add valuable information about changes due to waterlogging stress and should be considered in future experiments. Further reductions in biomass accumulation following draining may be due to damage associated with post anoxic shock (Setter and Waters 2003).

The nutrient data (Table 5.1) show that 20 d of waterlogging caused significant perturbations in plant nutritional status. Pardales *et al.* (1991) found that the sorghum root system was much smaller in waterlogged plants and that recovery from waterlogging was brought about by the resumption of nodal root growth. Seminal roots showed no sign of active regrowth during waterlogging. Malik *et al.* (2002) also found the seminal root system in wheat stopped growing upon waterlogging, instead adventitious roots commenced growth and resumed elongation after plants were drained. Assuming lucerne and wheat roots respond

similarly to waterlogging and the fact that a much-reduced root system is responsible for nutrient and water transport in a recovering plant, it is understandable that lucerne productivity will suffer even after a period of recovery. Therefore, it can be suggested that incomplete recovery of lucerne biomass after 16 d of stress withdrawal is at least partly due to the earlier disintegration of the taproot and associated reduction of nutrient supply to the shoot.

5. 5. 2. Chlorophyll Fluorescence and Recovery

All measured fluorescence parameters (Fv/Fm, qP, qN, NPQ and ETR) showed a near complete recovery by the end of the experiment (Fig 5.1; panel D, E, and F; Figure 5.2; panel C and D). It needs to be stressed, that fluorescence was measured essentially on the same leaves, that had become chlorotic while being waterlogged but that re-greened upon aeration. It is also important to stress that in control leaves, Fv/Fm values remained at around 0.8 level (Figure 5.1; panel D) when measured at the end of the recovery period, suggesting that leaf ageing and/or any other phenological aspects were not an issue. No visual symptoms of stress (red stem pigmentation due to anthocyanin production and leaf chlorosis) were present in our experiments 16 days after stress withdrawal, suggesting that photosynthetic performance of plants was repaired to pre-stress levels. Although, based on leaf gas exchange measurements, some authors showed that some plants recover their photosynthetic activity quickly after removal of waterlogging (Ahmed *et al.* 2002; Davies *et al.* 2000a), the only reports dealing with kinetics of chlorophyll fluorescence recovery after anaerobiosis is given in Haldimann and Strasser (1999). These authors observed a 10-20% reduction in the maximum quantum yield of photosystem II (Fv/Fm) when excised leaves of pea were subjected to anaerobiosis, and recorded a time-dependent recovery of Fv/Fm from the anaerobiosis-induced change, once leaves were returned to the air. This is the only evidence of recovery of chlorophyll fluorescence from waterlogging injury in the literature that I was able to find, and, therefore, the data appear to be the first *in planta* measurement of this sort. The re-greening of previously chlorotic lucerne has been reported before (Teutsch and Sulc 1997). The duration of the

stress is obviously a critical factor for the success of recovery. A much longer (more than 20 d) timeframe of stress would ultimately lead to the inability to recover and cause the crop to die. The question remains, what are the critical factors of survival. It appears that during recovery, the plant had to direct its energies into renewed pigment production and re-establishment of the photosynthetic apparatus, as a priority before growth could resume.

5. 5. 3. Nutrient Dynamics

Waterlogging causes a cessation of root growth (Trought and Drew 1980a) and this together with a drop in root respiration causes a drastic reduction in nutrient uptake and transport of mineral nutrients to the shoots within a very short time of waterlogging (Barrett-Lennard *et al.* 1999; Colin-Belgrand *et al.* 1991). Boem *et al.* (1996) found that brief periods of waterlogging caused a significant decline in uptake of N, P, K and Ca in canola. Atwell and Steer (1990) reported much reduced N, P and K concentrations in waterlogged maize shoots. These results are consistent with my data (Table 5.1). Stieger and Feller (1994) also found reduced P, K and Mg concentration in wheat shoots that had been waterlogged for up to 38 d, while Ca concentration in the vegetative parts of wheat were hardly influenced by waterlogging, concentrations in the grains were lower. Low Ca mobility in the phloem is implicated to explain this fact (Stieger and Feller 1994). In lucerne leaves, however, I found Ca concentrations to be affected by waterlogging; concentrations in waterlogged leaves were 80% lower than in control. This is supported by Sharma and Swarup (1989), who also found that Ca content (as well as N, P, K, Mg) was significantly reduced in shoot and root tissue of wheat after waterlogging. These authors conclude that even a very brief period of waterlogging adversely affected growth, yield and mineral composition of wheat due to decreased uptake of nutrients. Tarekegne *et al.* (2000) recorded a significantly reduced Cu, Zn, P and K nutrient uptake in waterlogging-sensitive wheat genotypes compared to more tolerant genotypes. The authors concluded that selection of genotypes with enhanced ability to overcome waterlogging-induced nutrient deficiency, particularly P an Zn

deficiency, should be encouraged and would lead to improved productivity in waterlogged soils.

5. 5. 4. Increased Sodium Uptake

A low concentration of oxygen in the rooting medium decreases the selectivity of K^+/Na^+ uptake in favour of Na^+ and retards the transport of K^+ to the shoots (Armstrong and Drew 2002). In my experiments, both leaf and stem tissues had a more than two-fold and three-fold increase of sodium, whereas sodium in the root tissue of the waterlogged plants had reduced to half of the control concentrations. Barrett-Lennard *et al.* (1999) recorded substantially increased net rates of Cl^- uptake into shoots and demonstrated increased Na^+ and Cl^- concentrations in the expanded leaf in wheat subjected to the combined stresses of salinity and waterlogging conditions. A so called "memory effect", when enhanced shoot transport of Na^+ remains present for many days even after short-term (1 h) oxygen deficiency, is known from the literature (Brauer *et al.* 1987). This could be one reason for the increased net fluxes of Na^+ into the shoot tissue. The precise ionic mechanisms involved remain to be elucidated. Barrett-Lennard *et al.* (1999) hypothesized that hypoxia increases permeability of root membranes to Na^+. However, as there are multiple pathways of Na^+ in roots (Maathuis and Amtmann 1999), it is not clear what specific Na^+ transporters are involved. Also, the absolute values of accumulated Na^+ in leaves remained relatively low despite two to three-fold increase in stems and leaves (Table 5.1) and, therefore, were not high enough to cause the observed decline in PS II photochemistry. More likely, deficiencies in N, P, K, Mg and Ca were major contributors. Results of Sharma and Swarup (1989) supported the view that the damaging effects of waterlogging in wheat were not due to toxic levels of Na and Fe but rather the reduced concentrations of N, P, K, Ca and Mg. The observation that Na^+ concentrations in waterlogged leaf tissue are increased, however is important to note, considering that waterlogging and salinity stress often coincide in Australian agricultural soils. With increased permeability, the likelihood of toxic Na concentrations in plant tissues under these conditions is likely.

5. 5. 5. Deficient Nutrients and their Effect on Photosynthetic Capacity

In this study, many of the nutrient concentrations in the stressed lucerne plants fell either into the deficient or marginal range according to Reuter and Robinson (1997). Nitrogen concentrations in waterlogged leaf tissue were 2.3% of DW and are considered deficient; phosphorous concentrations were marginal; K concentrations reached critical levels; Mg leaf concentrations at 0.29% DW were borderline marginal, close to deficient; Cu concentrations were deficient and Zn marginal. All these nutrients are crucial for leaf photosynthesis.

Magnesium is an important component of chlorophyll as well as being involved in the activation of a large number of photosynthetic enzymes, carbohydrate partitioning and phloem loading (Cakmak *et al.* 1994; Laing *et al.* 2000). A 30 to 40% reduction in Mg content in leaves observed in our experiments (Table 5.1), therefore, is likely to be responsible for observed leaf chlorosis as waterlogging progressed. Deficiency in Mg will affect the function of chloroplasts and the electron transfer in photosystem II (McSwain *et al.* 1976); this is reflected in the chlorophyll fluorescence data, which shows a reduction in ETR in waterlogged plants (Figures 5.1, panel B).

Other macronutrients are also directly involved in photosynthesis. Nitrogen is a key constituent of photosynthetic enzymes; it is found in the photosynthetic complex, especially in the protein Rubisco (Lambers *et al.* 1998). Potassium is a balancing ion for maintaining pH gradients across the thylakoid membrane (Pottosin and Schonknecht 1996). Being an activator of many photosynthetic enzymes, it is involved in photosynthetic CO_2 assimilation (Lauer *et al.* 1989) as well as regulating phloem transport. K is also a major osmoticum in living cells, responsible for the expansion growth of plant tissues and, thus, ultimately contributing to biomass accumulation. Photosynthetic efficiency per unit of chlorophyll is much lower in phosphorus deficient leaves (Lauer *et al.* 1989). Phosphorous is a major constituent of many key molecules contributing to leaf growth and photosynthetic performance as well as control such crucial enzymatic reactions as glycolysis, starch synthesis and CO_2 fixation (Marschner 1995).

Cu is a constituent of the chloroplast protein plastocynin as well as serving as part of the electron transport system linking PS I and PS II (Jones 1998). The reduction of Cu by 50% in waterlogged plants is potentially contributing to the reduced efficiency of PS II in waterlogged lucerne. Iron (Fe) and Mn are also important components in the functional units of photosynthesis. Both Fe and Mn are reduced in waterlogged leaf tissue, however, according to values given in Reuter and Robinson (1997), Mn levels at 54 mg/kg in leaf tissue were still adequate and Fe concentrations between 45-60 mg/kg are also considered adequate. Measured values for Fe in leaves of stressed plants were 74 mg/kg and in the stem values were around 44 mg/kg. It appears as though Fe concentrations were not limiting for photosynthetic functioning. Fe concentrations in the root tissue, however, are increased fivefold in the stressed plants. It is known that the reduction of insoluble Fe (III) oxides to Fe (II) is stimulated in waterlogged soils (Ponnamperuma 1972) and Fe (II) is readily taken up by the plant into the root tissue. A prerequisite for a healthy plant is an optimally functioning photosynthetic apparatus. A reliable measure of the health of the plant therefore is the assessment of its level of function. Surprisingly, with a few exceptions (Wagner and Dreyer 1997; Webb and Fletcher 1996), chlorophyll fluorescence has not been widely used to assess plant responses to waterlogging.

5. 5. 6. Implications for Recovery

In some previous work (Smethurst and Shabala 2003) I studied physiological responses of various lucerne cultivars to 16 d waterlogging. Although maximal quantum efficiency of PSII (Fv/Fm) progressively declined during waterlogging stress, no significant difference between four cultivars was measured. In addition to that parameter, in the present study, I also analysed the parameters of photochemical quenching (qP) and non-photochemical (qN and NPQ) quenching, during both waterlogging and subsequent recovery stages. These data suggest that while there is no clear cultivar response, there was a significant variation in measured parameters between individual plants in terms of photosynthetic (chlorophyll fluorescence) responses (Figure 5.4). This fact points to the possibility of identifying individuals with superior stress coping

mechanisms and highlights the applicability of chlorophyll fluorescence measurements as a convenient non-destructive tool for rapid assessment of a large number of individual plants. Before any definite conclusions about these individuals or genotypes as to their performance in waterlogged conditions can be made, however, extensive field-testing is required to validate any conclusions reached from glasshouse trials.

The recovery of the plants after waterlogging stress was removed was remarkable and better than expected. Three weeks of waterlogging is a reasonably severe case of waterlogging and generally leads to significant damage to lucerne pastures. The recovery found here suggests that it is not waterlogging alone that is limiting lucerne under these conditions in field situations. The increased uptake of Na found here could be an issue in areas with combined stresses of salinity and waterlogging and will be investigated further. Another aspect that could influence the recovery of plants is the cutting or grazing treatments prior to waterlogging stress. Plants that were regrowing from grazing or cutting prior to waterlogging would have low energy reserves and may not be able to recover so this factor will also be investigated.

NPQ measures the efficiency of thermal energy dissipation (Maxwell and Johnson 2000) and thus the amount of energy not used in photochemistry. NPQ was used to assess the photochemical damage caused by the temporary stress of waterlogging and the degree of recovery of this parameter following free drainage. NPQ as well as other fluorescence parameters recovered to near pre-stress levels. It appears that NPQ may be even more sensitive than Fv/Fm to measure waterlogging effect on leaf photosynthetic performance in lucerne, at least under our experimental conditions. This is also consistent with previous work in our lab when chlorophyll fluorescence parameters were used to screen plants for salt tolerance (Shabala *et al.* 1998).

5. 6 CONCLUSIONS

Plant nutrient status was adversely affected by waterlogging, with concentrations of nearly all macronutrients and micronutrients being significantly

lower than in drained controls. Concentrations of nutrients in leaves of recovered plants, however, were mostly not significantly different to those of control plants (Table 5.2). Although relative dry matter production of lucerne after recovery was less than in controls, near complete recovery of the photosynthetic apparatus was observed. Taken together these results may reflect both the smaller capacity of assimilation in previously waterlogged plants and the plant's need to re-direct available nutrient and assimilate pools to repair the damage to the roots and the photosynthetic apparatus. Although no significant genotypic difference was observed between the four cultivars used, a wide variation in chlorophyll fluorescence characteristics was measured from individual plants. If this variation of chlorophyll fluorescence parameters does correlate with waterlogging tolerance of individual plants then chlorophyll fluorescence might be a useful selection tool for "outstanding individuals", which could then be utilised by a breeding program. Any genetic selection based on glasshouse experiments, will have to be confirmed by extensive field trials, which simulate natural conditions more closely.

CHAPTER 6

SALINITY – GLOBAL CHALLENGE AND PLANT RESPONSES

6. 1. INTRODUCTION: –WHAT CONSTITUTES A SALINE SOIL?

6. 1. 1. Salinity

Salinisation is the process of increasing concentration of total dissolved salts in soil and water, either due to natural forces or human induced actions, called primary and secondary salinisation respectively (Ghassemi *et al.* 1995). Secondary salinisation is the result of mobilizing salt from the soil or groundwater by adding extra water to the system through irrigation or land clearing. When the watertable comes close to the soil surface, water evaporates, leaving salt behind thus causing salinisation (Barrett-Lennard 2002; Ghassemi *et al.* 1995; Lambert and Turner 2000). These salts can move in every direction, laterally and vertically, and can cause salinisation of whole water catchments.

Saline soils vary widely in their chemical and physical characteristics, salt dynamics and hydrology. The variables include salt source, concentration of salts, spatial distribution of salts in the soil profile, soil pH, clay content, organic matter content, nutrient status, water relations, temperature and soil toxicities. These factors have implications for the management of saline soils and for the breeding of salt resistant crop varieties (Staples and Toenniessen 1984).

If a soil contains sufficient soluble salts to adversely affect plant growth and/or land use, it is considered to be saline. High levels of salt may result in reduced plant productivity and may in extreme cases lead to a complete failure of crop establishment. Generally, when the saturation extract (solution extracted from a soil at its saturated water content) of soil has an electrical conductivity (EC) of > 4 deci Siemens (dS) m^{-1} the soil is considered saline (Greenway and Munns 1980; Lambert and Turner 2000).

Salinity is a widespread problem in many parts of Australia, where it is caused by an imbalance between rainfall and transpiration in dryland cropping systems (Cocks 2001). It has to be remembered that not only the land is affected, but river-systems and wetlands as well. Urban water supplies and infrastructure are also at risk. It is estimated that in Western Australia alone 450 plant species as well as many invertebrates are at risk of extinction. Many water birds are disappearing from Western Australia's wheatbelt. Collectively these consequences of salinity represent a far greater cost to the nation than does land degradation by itself (Cocks 2001).

6. 1. 2. Sodicity

It is estimated that more than 60 % of the 20 million ha of Australia's cropping soils is sodic and farming practises on these soils are largely based on dryland conditions. A soil containing sufficient exchangeable Na in relation to the other exchangeable base cations to adversely affect soil stability, plant growth and/or land use is considered sodic. Sodicity is caused by Na^{+} displacing Ca^{2+} and attaching to clay in the soil to such a concentration that it affects soil structure. The sodium weakens the bonds between soil particles and causing the clay to disperse making the soil water cloudy. The clay particles then move through the soil clogging pores and reducing drainage. Sodic soils are prone to erosion due to the fine particle size (Isbell *et al.* 1983; McKenzie 2004). Sodic soils may be improved by the addition of gypsum. Strongly sodic soils are considered to be those with an Exchangeable Sodium Percentage (ESP) of 15% or more.

6. 2. THREAT TO THE ENVIRONMENT

6. 2. 1. Introduction

Salinity is a major factor limiting agricultural productivity in large areas worldwide. Salinity is a particularly common problem in arid and semi-arid regions, where insufficient precipitation fails to flush accumulated salts from the crop root zone (Bresler *et al.* 1982) and the use of poor quality irrigation water exacerbates the problem. This process frequently causes progressive salinisation,

which is difficult to control. Increased salinisation of arable land is expected to have devastating global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang *et al.* 2003). The combined effect of poor quality irrigation water and the problem of rising water tables due to leakage and subsequent poor drainage causes gradual build up of salts in the upper soil profile and leads to land degradation due to accumulated salts (Szabolcs 1994). The affected land is difficult to reclaim (Husain *et al.* 2003).

6. 2. 2. The Extent of the Problem

Salinity especially affects developing countries where it forces the population to migrate to other areas, since the technology to combat salinity is too costly to be implemented (Asins *et al.* 1993). The estimate of the global extent of saline soils ranges between 400 and 950 M ha, and it has been calculated that one third of the 230 million ha under irrigation is affected by salinity (Flowers and Yeo 1997). Salt effected lands are often agriculturally productive areas under irrigation and the associated spread and extent of saline soils across the world is alarming. India and Pakistan as well as Australia have large areas affected by salinity; in Pakistan nearly 30% of cultivated land is salt-effected (Flowers and Yeo 1997). Other regions in the world include China, California and many countries on the African continent. In Western Australia alone two million hectares of land in the south-west of the state are salinised; all of the streams originating in agricultural catchments here are saline, only three freshwater ecosystems are left (Lambert and Turner 2000). In Australia more than 5.6 million ha of land have already succumbed to salinity and the predictions are that saline land will increase six-fold according to the National Land and Water Resources Audit (Choinski *et al.* 2003; Cocks 2003). 51% of farms in Western Australia are affected by salinity. It is estimated that about 10^7 ha of irrigated land is abandoned each year world-wide, because of the adverse effects of secondary salinisation and alkalization (Flowers and Yeo 1997; Rawat and Banerjee 1998).

6. 2. 3. Dryland Salinity

Dryland salinity is caused by rising water tables mobilizing salt in the soil (Ghassemi *et al.* 1995). Substantial increases in the volume of water entering groundwater systems, due to large scale clearing of deep-rooted perennial native vegetation, have resulted in transport of salt to the upper soil profile, where it concentrates at low lying evaporation sites as well as in rivers and wetlands (Ward *et al.* 2001). Increased salt concentrations and waterlogging of soils pose a serious risk to biodiversity as they impinge on plant growth and survival (Zeppel *et al.* 2003). The problem of the ever-increasing spread of dryland salinity illustrates how a once well-intentioned act, believed to be entirely beneficial or innocuous has resulted in disaster (Hillel 1991).

6. 2. 4. Landclearing

Clearing of native vegetation for annual crops and pastures is recognized as a major cause of waterlogging and secondary salinity in southern Australia (Turner and Ward 2002). In the 1960s more than 400 000 ha of land was cleared annually for agriculture in Western Australia; land clearing continues at this alarming rate in Queensland, despite evidence that it will lead to salinisation (Blacklow 2003). Farming systems in Australia are notoriously leaky, i.e. farming practices based on annual crops and pastures leak more than 150 mm of precipitation to the watertable compared with natural vegetation, where only 0 to 10 mm of rainfall penetrates into the groundwater (Cocks 2001). Eventually groundwater, which in the Australian landscape is usually salty, rises to the surface bringing with it its load of salt. As a consequence large tracts of land are threatened by dryland salinity. Salinity not only threatens agricultural productivity but also has detrimental effects on ecological parameters, such as biodiversity and ecosystem health as well as on infrastructure and homes (National Land and Water Resources Audit 2001). It causes water catchments to become increasingly saline (Lambert and Turner 2000). This represents an enormous economic cost that is difficult to estimate. Salinity is largely a self-inflicted curse, which now demands our concerted effort to halt its devastation.

Clearing of agricultural land has resulted in significant changes to the surface and groundwater hydrology (Taylor and Hoxley 2003). In the wheatbelt of Western Australia it is estimated that average groundwater recharge and surface runoff have increased about ten-fold when comparing the current hydrology to that before land was cleared. Saline groundwater discharge and flood volumes have also increased significantly and may double over the next few years (Taylor and Hoxley 2003). This is because annual crops grow only for 5-8 months and leave the landscape largely devoid of transpiring vegetation for the remainder of the year. Furthermore, the roots of annual crops and pastures are confined to near-surface soil layers (Ward *et al.* 2001). On the other hand native vegetation draws water from as deep as 20-40 m (Nulsen *et al.* 1986).

6. 2. 5. The Challenge

The challenge for plant breeders is the development of crop plants more tolerant to saline environments. However, salinity of agricultural systems is often increasing rather than being stable, breeding for salt resistance therefore constitutes "chasing a moving target" (Flowers and Yeo 1997). Breeding for improvement in salt tolerance cannot be the only avenue for combating this problem, land management changes are of great urgency to protect, conserve and restore landscapes affected by salt (Flowers and Yeo 1995).

6. 2. 6. Ameliorating Salt Affected Land

The accelerated salinisation of arable land in recent decades calls for urgent action. Development of salt tolerant plants not only for food production but also for land amelioration via reforestation and phase farming is important. Salt tolerant trees and shrubs have been planted to lower the water table (Barrett-Lennard 2002; Greiner 1997; Marcar *et al.* 1995). It has been shown that groundwater table can be lowered with extensive tree planting (Scott and Crossley 1996): groundwater fell to 150-200 mm in reforested area, compared to 100-125 mm under pasture.

Lucerne, as a deep-rooted perennial, has been advocated in phase farming systems to bring about a lowering of the water table (Crawford and MacFarlane

1995; Hirth *et al.* 2001; Humphries *et al.* 2004; Latta *et al.* 2002; Lolicato 2000; Passioura 1996).

Lucerne was shown to remove 50-100 mm more water from the soil profile than annual pasture, reducing average annual drainage beyond the root zone throughout a 5-year rotation from 45 mm to 17 mm (Ward *et al.* 2001; Turner and Ward 2002). Dunin (2002) reported transpiration by lucerne close to annual rainfall in the south West of Western Australia. These results suggest that lucerne has the potential to mitigate waterlogging/salinity stresses. Integration of persistent perennial species, woody and herbaceous, with traditional agriculture can provide satisfactory drainage control in environments prone to drainage constraints to ameliorate existing outbreaks of salinity (Dunin 2002).

Stabilizing hydrology with perennial crops instead of trees has the advantage of not locking up valuable agricultural land long-term but allowing intermittent cropping of annual cash crops once the perennial (such as lucerne) has exhausted excess water and nutrients in the soil profile. To select and breed more waterlogging and salt tolerant lucerne germplasm is therefore of great importance.

Plant functional responses and adaptations to salinity stress have been studied for decades and considerable advances have been made in identifying physiological reactions and mechanisms of salt tolerance. The following (and many other) reviews highlight what is known about salt stress on the whole plant level: Bernstein and Kafkafi 2002; Greenway and Munns 1980; Lazof and Bernstein 1999; Maas *et al.* 1977; Munns 2002; Munns and Termaat 1986; Shannon 1997; Yokoi *et al.* 2002. Ashraf and Harris (2004), Flowers and Yeo (1997), and Yeo (1998) provide detailed reviews on salinity tolerance mechanisms and related genetic traits. Furthermore, excessive salt loads adversely affect hydrology, biodiversity and soil structure and these aspects of salinity stress are addressed by Blacklow (2003), Cocks (2001), Ghassemi *et al.* (1995), Lambert and Turner (2000), Larcher (1995), and Zeppel *et al.* (2003).

Plant responses to salinity stress with emphasis on ion transport mechanisms is reviewed by Amtmann and Sanders (1999); Tester and Davenport

(2003); and Tyerman and Skerrett (1999); whereas Hasegawa *et al.* (2000) emphasized molecular mechanisms and signal transduction pathways in their review and the physiological consequences of altered gene expression due to salt stress.

Here I endeavour to summarise fundamental appraisals of these reviews and other recent literature on salinity stress in agricultural cropping systems and stress responses on cellular, tissue and whole plant levels. I also highlight the importance of ameliorating the accelerating salinisation of arable land via deep-rooted perennials such as lucerne to remove excess water from the soil profile in an attempt to combat waterlogging and the concomitant effects of salts moving up the soil column.

6. 3. WHOLE PLANT MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSES TO SALT STRESS

6. 3. 1. General: Salinity and Plant Yield

The mechanisms by which salinity affects biomass and yield can be via the osmotic effect or due to specific Na^+ toxicity (Munns 2002). The osmotic stress prohibits leaf formation, by inhibiting both leaf and tiller primordia in durum wheat. The Na^+ specific effect, on the other hand, affects the function and longevity of mature leaves, with high Na^+ concentrations accelerating desiccation and leaf senescence (Husain *et al.* 2003). Reduction in biomass, leaf area, shoot length, grain yield and other growth parameters due to salinity has been reported for many species, reviewed by Flowers and Yeo (1995), Maiti *et al* (2002), McKersie (1994b), and Wahome (2003). There are literally hundreds of references observing production losses due to salinity (Ashraf 2003; James *et al.* 2002; Rogers *et al.* 2003; Seemann and Critchley 1985, but the processes leading to this demise remain somewhat enigmatic.

6. 3. 2. Roots Responses

Root Growth and Architecture

The underlying mechanisms involved in the inhibition of root growth are still not clearly understood. Root tissue development adapts to altered regulation of water and solute transport under stress. Reduced growth in roots restricts the plant in the exploration of the growing medium and hence restricts the overall rate of water and nutrient uptake. The reduced supply of nutrients to the shoots leads to diminished growth in the leaves. This leads to a reduced ability of the shoot to supply assimilates to the roots and the growing tissues, which is likely to affect the whole plant development and survival, particularly if the plant is under long-term salinity stress (Munns and Termaat 1986).

Root architecture is influenced by salinity: root extension growth is most often severely inhibited by high concentrations of NaCl, but lateral root growth is less affected (Munns and Sharp 1993; Neumann 1995; Nonomi 1998). Root growth is limited to the region near the root tip. The root apical meristem and the elongation zone are of particular interest in salinity stress studies. Various studies have shown that the extent of tissue elongation is reduced under salinity stress; furthermore, the actual growth region is reduced under salinity stress. The stress-induced inhibition of root elongation may result from effects on the rate of cell division, rate of cell expansion, duration of cell growth or orientation of cell growth (Bernstein and Kafkafi 2002). Salinisation was shown to cause thicker but shorter roots (Huang and Redman 1995).

Exposure to increasing NaCl concentrations reduces root hair extension. Inhibitory effect of salinity on nodulation has been attributed to decrease in rhizobial colonisation and shrinkage and lack of root hair formation (Swaraj and Bishnoi 1999; Tu 1981). Poor root hair growth may be relieved by supplemental extracellular Ca^{2+} (Banet *et al.* 1996; Halperin *et al.* 2003; Lakshmi-Kumari *et al.* 1974; Shabala *et al.*2003).

Root Hydraulic Conductivity

Water moves through roots in response to a water potential gradient largely generated by transpiration. Root architecture dictates the flow of water within it. The quantity and rate of movement from the root to the shoot determine the ionic composition and concentration of solutes arriving at the shoot (Bernstein and Kafkafi 2002). Water uptake during growth maintains turgor pressure, which is the driving force for cell expansion. NaCl stress reduces hydraulic conductivity (Munns and Passioura 1984). Reduced hydraulic conductivity has been implicated in the root growth inhibition following salinisation. Water is osmotically held in salt solutions, so that with increasing salt concentration water becomes less and less accessible to the plant (Larcher 1995).

A sudden decrease in turgor pressure following salinisation is responsible for immediate, short-term inhibition of root growth. However, lowering of cell turgor does not appear to be the cause of long-term root growth inhibition (Munns and Termaat 1986). Long-term reduction of root elongation under salinisation seems to involve hardening of cell walls of expanding cells and influences the biosynthesis of cell wall polymers and wall metabolism (Bernstein and Kafkafi 2002).

Root Nutrient Acquisition

Uptake and transport mechanisms in the root inevitably affect ion composition in the shoot. Cell membranes are the major sites for controlling active and passive solute flux. Membrane structure and function of root cells are therefore of special interest in the study of solute transport under saline conditions (Bernstein and Kafkafi 2002). Under saline conditions, in many plant species Na^+ concentrations are several fold higher in roots than in shoots (Essah *et al.* 2003; Wahome 2003). Resistant plant species tend to have a reduced rate of translocation of ions from the root to the shoot and accumulate low amounts of salts in their above-ground tissues (Wahome 2003). Salinity causes reduced K^+ transport to the shoot (Flowers and Hajibagheri 2001) and accumulation of K^+ and Ca^{2+} in roots is strongly inhibited by salinity (Netondo *et al.* 2004). High K^+ vs

Na^+ selectivity of xylem loading in roots is a major determinant of tolerance to salinity (Bouraoui *et al.* 2001).

Long Distance Transport to Shoot

Xylem transport, phloem transport, root/shoot morphology and anatomy and transpiration are all affected by salt stress. Plants respond to salt stress with elevated ABA concentrations in roots and shoots with the possibility to interact with other hormones to coordinate whole plant responses to high salinity stress (Mulholland *et al.* 2003) and mineral imbalances in the soil (Jeschke and Hartung 2000). It has been shown that sos 1 (salt overly sensitive) Arabidopsis mutant has an up to 6-fold higher concentration of Na^+ in the xylem stream compared to the wildtype indicating that control of radial transport of Na^+ from the soil solution to the xylem vessels in the mutant is retarded (Nublat *et al.* 2001). Meristematic tissues are primarily fed by the phloem, which is low in Na^+ concentration but high in K^+ concentration. This mechanism helps prevent ionic imbalance in growing regions. This is an important protective mechanism since growing cells are more likely to be sensitive to disturbance of protein synthesis and other metabolic imbalances than mature cells due to their greater need for synthetic processes (Cramer 1997). Flowers and Hajibagheri (2001) argued that re-circulation in the phloem is only a temporary measure for removing the ions from the leaves as they remain available for re-transport to the shoot. Transpiration can influence the rate of ion transport to the shoot and the accumulation of ions in salt-stressed plants (Lauter and Munns 1987; Munns and Passioura 1984). Munns and Passioura (1984) suggested that ions in the transpiration stream of barley build up in the cell walls when ion content of the cells reaches a maximum. An accumulation such as this would cause loss of turgor then dehydration of cells and finally lead to cell death. These researchers concede, however, that in species more sensitive to salt than barley high ion concentrations within the cell may interfere with metabolic processes or damage membranes before ion uptake mechanisms become saturated, i.e. ions might never build up in the cell wall in salt sensitive crop plants.

6. 3. 3. Shoot Responses

Photosynthesis, respiration and carbon metabolism

Salinity affects photosynthetic performance (Brugnoli and Björkman 1992; Delfine *et al.* 1998; James *et al.* 2002; Meloni *et al.* 2003; Mickelbart and Marler 1996; Seemann and Critchley 1985; Velitchkova and Fedina 1998). Salt effects on photosynthetic processes fall into two major categories i) the response of stomatal conductance to salinisation of the plant (Chatrath *et al.* 2000; Ouerghi *et al.* 2000; Seemann and Critchley 1985) and ii) the effects of salt on the capacity of the plant for CO_2 fixation, independent of diffusion limitations ((Bethke and Drew 1992; Chatrath *et al.* 2000; 2003; Heuer 1997; James *et al.* 2002; Loreto *et al.*). For more detailed examples of photosynthetic responses to salt stress see Table 6. 1.

Dark respiration per unit leaf area in *Solanum muricatum* (Chen *et al.* 1999) increased due to NaCl salinity in the rhizosphere. Khavari-Nejad and Chaparzadeh (1998) found the same to be true for *Medicago sativa.* Rate of respiration and CO_2 compensation concentration increased in salt stressed pepino, *Solanum muricatum* Ait. (Chen *et al.* 1999). Others found that dark respiration did not seem to be affected by NaCl treatment (Marler and Zozor 1996; Mickelbart and Marler 1996). Enzymes of carbon metabolism are inhibited by excessive salinity and down-regulate metabolic pathways. Protein synthesis is also significantly inhibited by salt stress (Long and Baker 1986).

Table 6. 1.: Salinity and Photosynthesis

Species	Treatment	Major findings	References
Guava *(Psidium guajava* L.)	30 mM NaCl for 12 weeks	Net photosynthesis rate (P_N) reduced.	(Ali-Dinar *et al.* 1999)
Orange *(Citrus sinensis* L.)	60 mM of NaCl, KCl, $NaNO_3$ maintained for 8 weeks	NaCl and KCl caused net P_N and g_s to decrease, but $NaNO_3$ had no effect.	(Banuls *et al.* 1997)
Bell pepper *(Capsicum annuum* L.)	50, 100, 150 mM NaCl for 14 d	50 mM treatment had no affect on photosynthetic performance, but 100 and 150 mM NaCl caused up to 85% reduction of photosynthetic performance; chlorophyll content reduced only by 14% in highest salinity treatment.	(Bethke and Drew 1992)
Cotton *(Gossypium hirsutum* L.)	0, 26 and 55% natural seawater	Stomatal closure; at higher salinity levels also non-stomatal factors; chlorophyll fluorescence unaffected.	(Brugnoli and Björkman 1992)
Fodder oat *(Avena sativa* L.)	EC of 2, 4, 6, 8, 10 dS m^{-1} at flower initiation stage	P_N decreased with increasing salinity; at lower salinity levels stomatal limitations under higher salinity levels non-stomatal factors come into play.	(Chatrath *et al.* 2000)
Spinach *(Spinacia oleracea* L.)	1% (w/v) NaCl for 20 d	Photosynthesis reduced both by stomatal closure and changes in mesophyll structure.	(Delfine *et al.* 1998)
Durum wheat *(Triticum turgidum* L.)	150 mM NaCl for 4 weeks	g_s decreased; reduced CO_2 assimilation rate due to decreased g_s but with time also non-stomatal limitations.	(James *et al.* 2002)
Olive *(Populus euphratica* L.)	50 and 200 mM NaCl for 21 d	Net photosynthesis decreased for high salt treatment after 14 d.	(Ma *et al.* 1997)
Sapodilla *(Manilkara zapota* L.)	EC of 1, 12, 20 dS m^{-1} for 74 d	Net CO_2 assimilation decreased with increasing salinity.	(Mickelbart and Marler 1996)
Wheat *(Triticum durum and T. aestivum)*	50 and 100 mM NaCl for 21 d	Net CO_2 uptake was reduced at PPFD higher than 500 μmol $m^{-2}s^{-1}$ in sensitive cultivar, mostly because of stomatal closure.	(Oram *et al.* 2002; Ouerghi *et al.* 2000)
Bean *(Phaseolus vulgaris)*	0-150 mM NaCl for 10-14 d	gs declined, Ci reduced by up to 30%; photosynthetic CO_2 fixation reduced.	(Seemann and Critchley 1985)
Rice *Oryza sativa* L.)	0, 50, 100, 150, 200 mM NaCl, 6 h, isolated chloroplasts	Net photosynthetic rate drastically decreased under increasing salt stress, tolerant cultivar was less affected.	(Tiwari *et al.* 1997)

Stomatal limitations of photosynthesis

The reduced conductance to CO_2 diffusion due to stomatal closure accounts for much of the reduction in photosynthesis under water stress (Cornic *et al.* 1992) and moderate salt stress (Brugnoli and Björkman 1992). Sharma and Hall (1992) observed a reduction in CO_2 assimilation rate in salt-stressed *Sorghum bicolor* and speculated that this might be due to stomatal limitations. Decline in photosynthesis was attributed to stomatal conductance in isolated cowpea cells (Plaut *et al.* 1989) and in spinach (Delfine *et al.* 1998). When salt stress was relieved, both conductance and photosynthesis recovered. The inhibition of the Rubisco pool size and activity were evident only when salt accumulation in the leaf was high, which seemed to indicate that the contribution of carbon metabolism to photosynthetic inhibition in the early phase of stress is low (Delfine *et al.* 1998). However the decrease in CO_2 assimilation due to the combined effect of salinity and high light was not entirely due to stomatal conductance (g_s) as inhibition persisted even after g_s recovered completely (Sharma and Hall 1992). Di Martino *et al.* (2003) observed a decrease in photosynthesis when salt began to accumulate in the leaves – the decline was initially due to reduced stomatal conductance. Some other physiological changes occurred only after some time had elapsed; chlorophyll content was reduced after about 20 days of treatment, protein degradation took place at around day 43 of treatment, which coincided with a large loss of Rubisco. Photorespiration, which increased during salt stress, was also suggested to play a role in supplying metabolites to produce compatible osmolytes. Glycolysis and thus respiration increased to sustain the higher energy demand to confine salt to the vacuoles and to furnish carbon skeletons for the respiratory cycle.

Non-stomatal limitations

High concentrations of Na^+ and Cl^- within the plant might limit the activities of photosynthetic enzymes such as Rubisco. Seemann and Critchley (1985) found that independent of altered diffusional limitations, photosynthetic CO_2 fixation decreased in *Phaseolus vulgaris*. The quantum yield for net CO_2 uptake was also reduced by salt stress (Seemann and Critchley 1985). Quantum

yield was measured under saturating CO_2 concentrations thus precluding interference from stomatal closure resulting from salinity stress. In another study dealing with the effect of salinisation on photosynthesis in fodder oats (Chatrath *et al.* 2000), it was concluded that at high levels of salinity photosynthesis was not only limited due to reduced stomatal conductance but also influenced by the reduction in the biochemical photosynthetic capacity, because at this level of salinity a significant reduction in carboxylation efficiency (P_N/C_i) was measured. Everard *et al.* (1994) confirmed that at high salinities (300 mM NaCl) carboxylation capacity and electron transport were the prevailing limits in *Apium graveolens* L.

Reductions in photosynthesis associated with non-stomatal inhibition of photosynthesis by salt have been observed in a number of other salt-sensitive species (Delfine *et al.* 1999; Everard *et al.* 1994; Seemann and Critchley 1985), but these limitations are not well understood. The biochemical basis for this photosynthetic reduction due to salt stress might be explained by either a change in the concentration of photosynthetic machinery and or a change in the efficiency with which the machinery operates.

Reports on chlorophyll fluorescence responses to salt stress vary (see Table 6. 2.); the general trend being that at low levels of salt stress Fv/Fm is not affected, but at higher levels of salinity and more sustained exposure (Corney *et al.* 2003; Everhard *et al.* 1994; and James *et al.* 2002) Fv/Fm declined significantly. Excised leaves, however responded with a decrease in photosystem II efficiency (Belkodja *et al.* 1999).

Table 6. 2.: Salinity and Chlorophyll Fluorescence

Species	Treatment	Major findings	References
Barley *(Hordeum vulgare L.)*	Excised leaves incubated in 100 mM NaCl	Fv/m decreased from 0.84 to 0.72 after 2h incubation.	(Belkodja *et al.* 1994)
Barley *(Hordeum vulgare L.)*	Salinity of irrigation water ranged from 2-18 dS m^{1}, fluorescence readings on intact and excised leaves	No decrease in photosystem II efficiency in the field, but Fv/Fm of excised leaves decreased.	(Belkodja *et al* 1999)
Eucalypts (*Eucalyptus camaldulensis*)	200 mM NaCl for 60 d	Variable fluorescence, F_{ds} (light-adapted) started to decline at d 30 and declined incrementally to d 60.	(Corney *et al.* 2003)
Spinach (*Spinacia oleracea L.*)	1% (w/v) NaCl for 13 d	No affect on photochemistry; FvFm remained the same as control.	(Delfine *et al.* 1998)
Celery (*Apium graveolens* L)	25, 100 and 300 mM NaCl; measurements taken 4-5 weeks after treatment	Significant reduction of Fv/Fm only at 300 mM NaCl.	(Everard *et al.* 1994)
Durum wheat (*Triticum turgidum* L.)	150 mM NaCl for four weeks in hydroponic system	Fv/Fm declined only at latest harvest likely due to toxic ion effects (salt-induced photo-damage); NPQ increased indicating thermal dissipation of excess light energy.	(James *et al.* 2002)
Roses (*Rosa hybrida* and *R. manetti*)	0, 20, 50, 100 mM NaCl, 120 d in glasshouse under low and high irradiance	Fv/Fm decreased only slightly after 4 months, but under high light decreases were significant, also in control plants, Fv/Fm not a sensitive parameter for salt tolerance.	(Jimenez *et al.* 1997)
Rice (*Oryza sativa* L.) cultivars	0, 30, 50 mM NaCl; Fv/Fm measured every 3 days for 30 d	After 18 d no difference between cultivars, although Fv/Fm decreased over time, after 30 d significant cultivar differences (P = 0.05) to as low as 0.729; suggested that Fv/Fm is not a good screening tool.	(Lutts *et al.* 1996)

Genetic variation in photosynthetic performance

Genotypic responses to salt stress have been observed for many species, e.g. for olive (Loreto *et al.* 2003); citrus (Banuls *et al.* 1997); wheat (James *et al.* 2002); cotton (Meloni *et al.* 2003); and eucalyptus (Rawat and Banerjee 1998). In a study by Ali-Dinar *et al.* (1999) red and white guava showed distinctly different responses in photosynthetic capacity when subjected to the stress of salinity. Ouerghi *et al.* (2000) also found genotypic differences between two wheat species subjected to saline conditions. Dionisio-Sese and Tobita (2000) found similar results in rice, although, even the tolerant cultivar showed some reduction in carbon assimilation, but not as severe a reduction as the sensitive cultivars.

Theory and application of chlorophyll fluorescence for studying abiotic stresses such as salinity has been discussed in Chapter 2. Since visible signs of salinity stress are rather late manifestations of the stress and are difficult to quantify, physiological parameters (such as chlorophyll fluorescence) are being sought as suitable indicators for response mechanisms of salt tolerance. Salt sensitivity is associated with increased shoot Na^+ accumulation, decreased PSII photochemical efficiency and enhanced non-photochemical quenching in rice (Dionisio-Sese and Tobita 2000). Therefore analysis of quenching parameters may reveal how photosynthetic activity is affected by salt stress. It might be advisable to use quenching parameters as indicators of stress. Shabala *et al.* (1998) found that when NaCl was above 50 mM NaCl, a progressive decrease in yield, Y, and in the coefficient of photochemical quenching, qP, in illuminated leaf samples of corn occurred. At the same time, the non-photochemical quenching element of fluorescence quenching qN increased (Shabala *et al.* 1998). These researchers concluded that fluorescence quenching parameters constitute the most sensitive fluorescence characteristics for measuring salinity sensitivity in corn. Similar conclusions were reached for barley (Belkodja *et al.* 1999), for spinach (Delfine *et al.* 1999) and rice (Dionisio-Sese and Tobita 2000).

6. 3. 4. Photosynthate Partitioning

Salt-stressed plants initially maintain turgor mainly by accumulating organic and inorganic solutes in plant tissues (mainly in leaves) through osmoregulation. Osmotic adjustment is aided by sequestering of these ions into the vacuole. As a result maintenance energy costs of plant cells are likely to increase to accommodate NaCl evacuation from the cytosol to the vacuole. The increased use of metabolic energy (ATP) to evacuate ions from the cytosol is likely to reduce the carbon use efficiency of cells grown in NaCl (Binzel and Reuveni 1994). The lowered osmotic potential in the vacuole maybe also balanced by organic solutes such as sugars, glycine betaine, and proline in the cytoplasm. Depending on the degree of salt tolerance in individual species, plants will eventually reach concentrations of Na^+ and Cl^- in the transpiring leaves that are no longer able to be compartmentalized in the vacuole, but instead build up rapidly in the cytoplasm and inhibit enzyme activity (Munns 2002). The metabolic cost associated with the physiological processes associated with adaptation to salinity, including the diversion of carbon into osmotic solutes, could result in a depletion of carbon supplies necessary for cell expansion and cell division (Binzel and Reuveni 1994).

6. 3. 5. Other Factors Affecting Performance under Saline Conditions

Nutritional disorders

Many researchers report deficiencies in key nutrient ions in various plant tissues due to salinity, such as magnesium, phosphorus, and potassium (e.g.: Kaya *et al.* 2001; Koyro 2000; Netondo *et al.* 2004; Shiyab *et al.* 2003). Nitrogen deficiency might occur in plants exposed to NaCl due to Cl^- interfering with NO_3 fluxes into the root, but data are not conclusive (Munns and Termaat 1986).

According to Grattan and Grieve (1992), salinity can interfere with acquisition of mineral nutrients by disrupting mineral relations of plants by reduction of nutrient availability through competition with major ions (i.e. Na^+ and Cl^-) in the substrate. These interactions often lead to Na^+-induced Ca^{2+} and/or K^+ deficiencies and Ca^{2+}-induced Mg^{2+} deficiencies (Sairam and Tyagi 2004).

Saline conditions are characterized by low nutrient-ion activities and extreme ratios of Na^+/Ca^{2+}, Na^+/K^+, Ca^{2+}/Mg^{2+} and Cl^-/NO_3^-, nutritional disorders can develop and crop growth may be reduced. Gypsum can correct the Ca^{2+} deficiency and ameliorate the deleterious effect of Na. Salinity can aggravate Mg deficiency (El-Katony 1998; Murillo-Amador *et al.* 2002).

Hormonal changes

Salinity stress response is multigenic and various processes are involved such as ion transport, compartmentation of harmful ions and antioxidant defence mechanisms. Production and distribution of compatible solutes, polyamines and reactive oxygen species is also affected by salt stress. Genes are involved in this regulatory process, which might up-regulate hormones like abscisic acid (ABA), jasmonic acid (JA) and compounds such as polyamines (Moons *et al.* 1995; Moons *et al.* 1997). Theses chemical compounds are possibly involved in the transduction of stress signals to improve salinity stress tolerance in certain species (Chattopadhayay *et al.* 2002; Kao *et al.* 1997; Li *et al.* 2002; Maiti *et al.* 2002; Sairam and Aruna 2004). Munns (1993) argued that salts accumulated in plants do not directly control plant growth by affecting turgor, or photosynthesis. Rather, the increasing concentration of salt in old leaves hastens senescence, and the loss of these leaves affects the supply of assimilates or hormones to the growing regions and thereby affects growth. Expression levels of ethylene receptors are modulated by NaCl stress in Arabidopsis (Zhao and Schaller 2004). Application of gibberellic acid (GA3) was found to significantly increase photosynthetic capacity and stimulated vegetative growth (Ashraf *et al.* 2002). Aldesuquy and Ibrahim (2001) also found that the application of growth bioregulators (gibberellic acid and indole-3-acetic acid (IAA)) appeared to mitigate the effect of salt stress on wheat productivity.

6. 4. CELLULAR RESPONSES TO SALT STRESS

6. 4. 1. General

Growth, morphology, anatomy and physiology in roots as well as shoots are all affected by salinity. The reactions to salt stress are expressed on a whole plant and on a cellular level, however whole plant reactions are more complex than those elicited by cells in suspension and the communication between different tissues is required for a complete environmental stress response (Adams *et al.* 1992). One of the key factors of salt tolerance is the plant's ability to maintain cytoplasmic ionic homeostasis, therefore understanding membrane transport regulation is paramount (Yeo 1998).

6. 4. 2. Ionic Relations and Membrane Transport Activity

Plant salt tolerance operates at various levels, including the cellular level, such as ion sequestration into vacuoles or ion exclusion at plasma membranes. Maintenance of ionic homeostasis in the cytosol and the vacuole are complex. They involve the coordination of pumps and channels that adjust ion transport across plasma membrane, tonoplast and organellar membranes (Hedrich and Schroeder 1989; Tester 1990). Coordination of the transport activity is a prerequisite for intracellular ionic homeostasis in saline environments (Binzel and Reuveni 1994). The similarity of the hydrated ionic radii of Na^+ and K^+ makes it difficult to discriminate between them and this is a major factor of Na^+ toxicity (Blumwald 2000). Only 10 years ago there was little knowledge about ion channel activity *in planta* with regard to different channel types, their properties and their localization in different plant tissue (Véry and Sentenac 2002). Since then a range of biophysical and molecular techniques have allowed a greater understanding of different channel types, and have identified some of their functions (Bohnert and Sheveleva 1998; Bray 1997; Elphick *et al.* 2001; Franklin and Zwiazek 2004; Hasegawa *et al.* 2000; Klobus and Janicka-Russak 2004; Shabala 2000; Tester and Davenport 2003; Volkov *et al.* 2004; Zhu *et al.* 1998).

Under saline conditions roots must acquire essential nutrients such as N, P and K, while excluding toxic ions such as Na^+. Metabolic toxicity of Na^+ is

largely the result of its ability to compete for K^+ binding sites essential for cellular function. More than 50 enzymes are activated by K^+ and Na^+ cannot act as a substitute in this role (Tester and Davenport 2003). High levels of Na^+ thus disrupt many enzymic processes in the cytoplasm. Protein synthesis requires high concentrations of K^+ since it is needed for the binding of tRNA to ribosomes (Blaha *et al.* 2000). Na^+ can inhibit enzyme function by binding to inhibitory sites or directly by displacing K^+ from activation sites (Serrano 1996). Damage caused by elevated levels of Na^+ in the cytoplasm is manifold and still requires closer scrutiny and characterisation. Because neither Na^+ nor K^+ are incorporated into other molecules, cytoplasmic concentration is determined by a combination of influx and efflux transport activities.

Plasma membrane transport

Maintaining high K^+/Na^+ ratios in the cytoplasm is a key feature of salt tolerance. One of the important questions to be asked is, how does modulation of the activity of the different channel types affect the ability of the plasma membrane to discriminate between K^+ and Na^+ (Amtmann and Sanders 1999). Ion homeostasis is achieved by the regulation of various membrane transporters and signal transduction pathways (Serrano and Rodriguez-Navarro 2001). A number of ion transporters have now been identified in cell plasma membrane that are likely to be responsible for Na^+ regulation (Tyerman and Skerrett 1999, Hasegawa *et al.* 2000). The model below on page 124 (based on Hasegawa *et al.* 2000) illustrates the main pathways for Na^+ uptake across the plasma membrane. They include:

- The major route for Na^+ uptake into the root is believed to be through non-selective cation channels (NSCC), either voltage independent (VICs) (Tyerman and Skerrett 1999; Tyerman *et al.* 1997; Amtmann and Sanders 1999) or weakly voltage dependent (Davenport and Tester 2000; Demidchik *et al.* 2002);

- High affinity potassium transporters, HKT1 (Laurie *et al.* 2002; Maathuis and Amtmann 1999); many of these HKT-type transporters seem to be sodium-specific and may regulate K^+ homeostasis in the presence of Na^+ (Rus *et al.* 2001);
- Low affinity cation transporter (LCT1) (Amtmann and Sanders 1999) may also function as Na^+ pathways;
- K^+-permeable channels: K-inward rectifying (KIR) and K-outward rectifying (KOR) channels (Maathuis and Amtmann 1999).

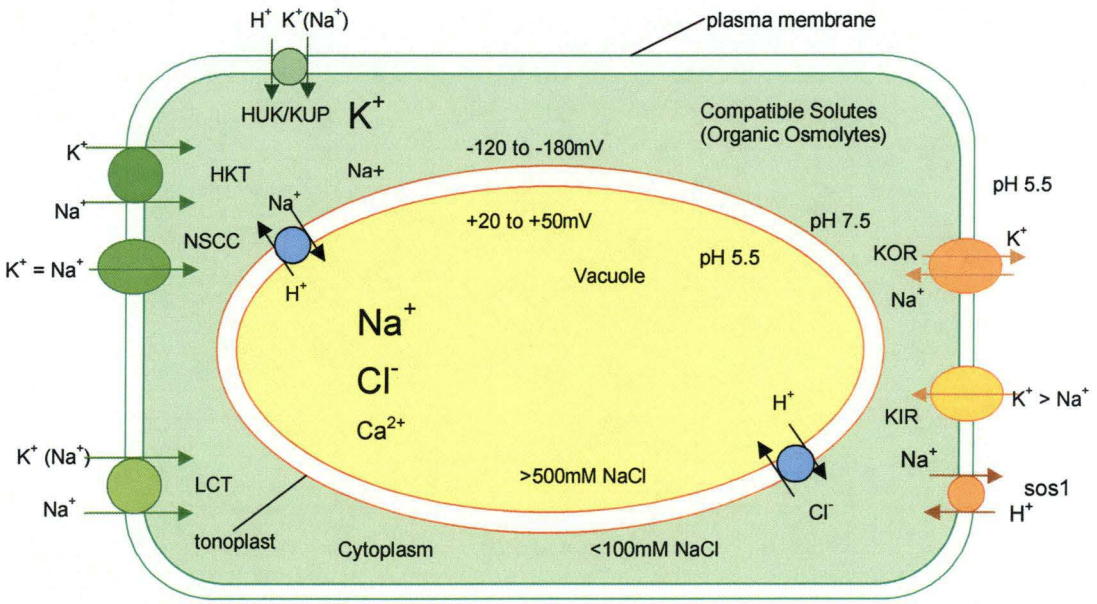

Figure 6. 1.: Major K^+ and Na^+ transport systems involved in regulation in ion homeostasis in the cytoplasm and K^+/Na^+ ratio. For further descriptions of channels and transporters refer to text.

Evidence is mounting that non-selective cation channels are the major pathway for Na^+ influx from the soil solution into the cytoplasm of root cells (Davenport and Tester 2000; Demidchik and Tester 2002; Maathuis and Amtmann 1999). This is mainly because these channels do not select strongly against Na^+ and are time and voltage independent (Amtmann and Sanders 1999). If a non-selective cation channel is a pathway for Na^+ in saline conditions then its downregulation would enhance salinity tolerance, but this approach to improving

salt tolerance may have undesirable consequences, as the function of these channels in non-saline conditions may be to facilitate entry of nutrients. Improving selectivity may well be a better approach than downregulation of channels, however, it is also a more difficult task (Yeo 1998). The absorption of Na^+ on the one hand is desirable as a cheap way of generating high internal osmotic pressure in response to high external osmotic pressure, thereby lowering cell water potential and sustaining turgor. On the other hand Na^+ is cytotoxic at concentrations greater than 100 mM (Amtmann and Sanders 1999).

Tonoplast transport

Compartmentation of Na^+ in the vacuole is another way to avoid cytoplasmic Na^+ overload and maintain the optimal cytoplasmic K^+/Na^+ ratio. Another advantage is that the cell turgor pressure is maintained during this process (Greenway and Munns 1983; Koyro 2000 and Lerner *et al.* 1994). Tonolast Na^+/H^+ antiporters are crucial for Na^+ compartmentation in the vacuole (see Blumwald *et al.* 2000, for a review). The steep pH gradient between vacuole and cytosol can be used to drive Na^+ from the cytosol to the vacuole (Binzel and Reuveni 1994; Blumwald *et al.* 2000). A ten-fold increase in Na^+ concentration was measured in the vacuole of sugar beet when a pH gradient of 1.5 units was established across the tonoplast (Barkla *et al.* 1990). For many plant species an induction of Na^+/H^+ antiporter activity in response to salt stress has been demonstrated and with it increases in tonoplast H^+-ATPase activity (Blumwald *et al.* 2000).

Xylem loading

The root is the primary sensor of salt stress, yet tissue and cell-type-specific functionality studies are rare (Kiegle *et al.* 2000). Solute transport into the root xylem involves flux from the symplast into the apoplast (Läuchli 1976). Xylem loading is mediated by thermodynamic passive transport mechanisms by both inward and outward rectifying ion channels (Wegner and de Boer 1997a; and Wegner and de Boer 1997b). The driving force is generated by H^+-ATPase. Salt stress can cause a significant alteration of ion concentrations in the xylem sap

(Jeschke 1992). The amount of K^+ translocated to the aerial parts of the plant is controlled by xylem parenchyma cells and is influenced by environmental stress factors such as soil salinity (De Boer 1999). The control of the K^+/Na^+ selectivity and discrimination during root xylem loading is considered an important mechanism for tolerance to salinity (Munns 2002) as tested in a wheat-rye hybrid (Bouraoui *et al.* 2001). When xylem sap was sampled on de-topped plants it was found that this hybrid had a high selectivity for K^+ over Na^+ when comparing concentrations of sap with concentrations in nutrient solution.

Salt stress (100 mM NaCl) has been shown to inhibit xylem loading of NO_3- and its subsequent transport to the shoot, which will cause N shortage in the shoot (Gao *et al.* 1996). Wegener *et al.* (1999) found that a tight electrical coupling exists between the cellular and tissue level in the root of intact maize plants and the resistance of the cellular (symplastic) space is much less than the resistance of the apoplast.

6. 5. MECHANISMS OF ADAPTATION

6. 5. 1. General

The ability of plant cells to maintain low cytosolic Na^+ concentrations is an essential process for plants to be able to grow in saline environments. Tolerance to salinity can also be achieved by Na^+ compartmentation into the vacuole, when ions have already accumulated in the tissue of the plant (Allen *et al.* 1994; Blumwald 2000; Greenway and Munns 1980). Salinity tolerance is a multigenic response and mechanisms as diverse as compatible solutes/osmolytes synthesis, reactive oxygen species and antioxidant defence mechanisms, ion transport and compartmentation might all be potential targets in molecular research into salt tolerance (Sairam and Aruna 2004). Each of these are potential ideal targets for genetic manipulation to increase salt tolerance in crop plants.

6. 5. 2. Whole Plant Level

To avoid excessive sodium accumulation the plant has various mechanisms at its disposal, but the effectiveness of these strategies varies with

species. Exclusion of Na^+ at the soil root interface is one potential strategy (Munns 1985); another strategy is the sequestering of Na^+ in the vacuole to prevent cytotoxicity.

Morphological adaptations

One of the mechanisms of salt tolerance observed in most species include the ability to increase total plant water content to dilute ion toxicity. Cell size increases and surface to tissue volume decreases and makes leaves more succulent (Flowers and Yeo 1986). Succulence occurs mainly owing to an increase of mesophyll cell size. Changes in root morphology and hydraulic properties might also be advantageous in saline environments (Maggio *et al.* 2001). These authors suggested that some salt tolerant species have been evolutionary selected for specific, yet unknown, morphological traits of the root system that influence ion and water transport. There is strong evidence that roots control the salt load to the shoot and therefore studying the regulation of transport processes as well as morphology and architecture of roots might shed more light on some components of the salt tolerance response. Other adaptations of coping with high salt concentrations in the soil solution are salt glands and bladders. Shoot ion concentrations can be regulated by secreting excess ions into theses organs (Gorham *et al.* 1985; Sobrado 2004). Excess salt can also be secreted into in salt hairs or microhairs, which are specialized structures of the epidermal layer, glandular secreting trichomes in halophytic grasses (Ramadan and Flowers 2004). Salt secretion appears to be an active process. Non-halophytic grasses also possess microhairs, but the question whether the glandular function is inducible for example in maize or other important agricultural crops, is still not known (Thomson *et al.* 1988).

Exclusion versus inclusion

Plant roots are able to efficiently differentiate between different ions in the soil solution such that most of Na^+ (up to 97%; Munns *et al.* 1999) are excluded from the xylem stream. This is an important first line of defence against salt stress. Ability to exclude Na^+ is an important factor in determining salinity

tolerance in many crop plants (Munns 1990). The latter may be achieved by either restricting Na^+ entry through the plasma membrane (Munns *et al.* 2002) or by active Na^+ extrusion from the cytosol via plasma membrane SOS1 Na^+/H^+ exchanger (Tester and Davenport 2003; Zhu 2003). Understanding the regulation of the specific Na^+ transport systems involved (NSCC and Na^+/H^+ transporter, respectively) are therefore central to understanding plant salt tolerance. Internal exclusion mechanisms involve processes such as sequestering salt ions in specialized tissue by removing them from the transport stream. Selective ion transport across the tonoplast enables Na^+ and Cl^- accumulation in vacuoles (Gorham *et al.* 1985) while at the same time maintaining adequate levels of K^+ in the cytoplasm until a threshold is reached when Na^+ accumulates to toxic levels in the cytoplasm and causes metabolic inhibitions (Lazof and Bernstein 1999). The ability to compromise successfully between osmotic adjustment, ion nutrition, and restriction of cytosolic Na^+ concentration and maintenance of energy pools is probably the key to salt-tolerance (Amtmann and Sanders 1999). There is some evidence that Na^+ exclusion from the shoot might be genotypically determined eg for maize (Hajibagheri *et al.* 1987) and *Triticum* x *Lophopyrum* derivatives (Schachtman *et al.* 1989).

Control of xylem loading

Besides cellular survival mechanisms, the whole plant also employs tissue specific survival strategies such as regulation of Na^+ delivery to the shoot by altering the loading of the xylem, retrieval from the xylem before Na^+ reaches the shoot, recirculation out of the shoot in the phloem and other strategies (Tester and Davenport 2003; Yeo *et al.* 1977). Research of tissue specific ion transport responses to salinity revealed that maize stele and cortical cells differed in their ion transport characteristics and that K^+-selective conductances possibly function as major components of the cation transport mechanism (Roberts and Tester 1995). It is hypothesized, that salt-stressed corn accumulates Na^+ in xylem parenchyma cells of mature roots and so removes Na^+ from the xylem vessels by selectively reabsorbing Na^+ (Yeo *et al.* 1977). The control of the K^+/Na^+ selectivity of ion secretion into the root xylem is considered an important factor in

salinity tolerance (Bouraoui *et al.* 2001). Na^+ can be removed from the xylem conduits in the roots and be exchanged with K^+ in adjacent cells. Entry of Na^+ into the leaf is thus reduced considerably (Atwell *et al.* 1999; Yeo *et al.* 1977). To avoid built up of salt in leaf blades, plants are able to exclude Na^+ ions from the xylem sap. Transpiration moves solutes throughout the plant, but selective exclusion enables salt restriction to the leaves as well as reproductive organs. Lohaus *et al.* (2000) reported that a significant portion of Na^+ and Cl^- imported into the xylem were exported by the phloem in maize. Apical tissues, such as root tips and shoot apices, import photo-assimilates, which are largely transported in the phloem. In tolerant plants such as barley Na^+ and Cl^- exclusion from phloem sap is very effective whereas in less tolerant plants these ions are more likely to accumulate in growing tissues.

Compartmentation in the whole plant

Water in the transpiration stream carries with it ions, which are eventually deposited in the leaves. Protection of young leaves is potentially one crucial element necessary for salt tolerance. Older leaves therefore often have higher concentrations of salt than younger leaves, develop necrosis sooner and may senesce prematurely (Atwell *et al.*1999; Munns *et al.* 1983). Preferential removal of Na^+ from solutions moving towards organs that need protection and deposition into older leaves can be done by solute cycling through phloem and xylem and selective deposition and removal of Na^+ in the xylem stream (Wolf *et al.* 1991). Any ions not excluded by the roots, will end up in the transpiration stream and soon build up to toxic levels in the shoot tissue (Gorham *et al.* 1985; Shannon 1997). Therefore control of transport of salt to the shoot is an important adaptive feature of salt-tolerant plants. This is also true for halophytes. The xylem sap of *Avicennia marina* was calculated to be only 9 mM NaCl although it grew in a substrate of ~500 mM NaCl (Ball 1988), demonstrating a very high exclusion capacity at the root. Jack pine is able to restrict NaCl ion uptake into the shoot and preferentially sequestering it into the root (Franklin and Zwiasek 2004).

Partitioning of toxic salts into various shoot tissues is an adaptive feature of salt including plants, e.g. preferential deposition into older leaves (Colmer *et al.*

1995), or into vegetative tissue rather than reproductive organs. Preferential accumulation of Na^+ has been observed in epidermal cells and was attributed to increased activity of non-selective cation channels in the plasma membrane of epidermal cells. High concentrations in bundle sheath cells also suggests that this cell type may be involved in minimizing Na^+ accumulation in the photosynthesising cells of the leaf mesophyll (Karley *et al.* 2000; Pitman *et al.* 1981; Steveninck *et al.* 1980).

6. 5. 3. Cellular Level

Na^+ exclusion mechanism

As no Na^+-specific pump has been found so far in higher plants, Na^+ exclusion from the cytosol is achieved by exchange for hydrogen ions across the plasma membrane. Sodium ions are exchanged for hydrogen ions across the membrane as membrane Na^+/H^+ antiporters take advantage of the proton gradient formed by plasma membrane H^+ pumps (Hasegawa *et al.* 2000; Mansour *et al.* 2003), thus coupling the H^+ movement along the electrochemical gradient to the extrusion of Na^+ against its electrochemical gradient (Blumwald 2000). The activation of such antiporters is likely to be responsible for enhanced salt tolerance. Exclusion of Na^+ ions at the plasma membrane is the main mechanism employed by salt sensitive plant species, while tolerant plants tend to accumulate Na^+ in the vacuole (Blumwald *et al.* 2000).

Vacuolar sequestration

In plant cells, the Na^+/H^+ antiport at the tonoplast provides a biochemical pathway to transport cytoplasmic Na^+ into the vacuole. Over-expression of a vacuolar Na^+/H^+ promotes sustained plant growth at high NaCl levels (Apse *et al.* 1999). Transgenic tomato plants over-expressing a vacuolar Na^+/H^+ antiport were able to grow, flower, and produce fruit in the presence of 200 mM NaCl and at the same time compartmentalize NaCl in the leaves and not into the fruit (Zhang and Blumwald 2000). While tomatoes transformed with NHX1 are reportedly able to exclude NaCl from the fruit, there may be a problem with a crop such as lucerne, where forage quality (for grazing) might be compromised on saline soils. Lucerne

on saline sites can be expected to have higher NaCl concentrations in the leaf tissue and therefore affect fodder quality. Vacuolar ATPase provides an energy source for transport of ions across the tonoplast. Membrane Na^+/H^+ antiporters take advantage of the proton gradient formed by these pumps to exchange Na^+ for H^+ across a membrane (Mansour *et al.* 2003).

Na^+/K^+ ratio and K^+ homeostasis

Accumulation of Na^+ and Cl^- in the cytosol to excessive levels will inhibit enzyme activity (Flowers and Yeo 1986). Membrane transport of ions for maintenance and re-establishment of homeostasis were reviewed by Hasegawa *et al.* (2000). Potassium is an essential co-factor for many enzymes and K^+ concentration of salt-adapted tobacco cells was able to be maintained at 80 mM in the cytoplasm, when vacuolar NaCl concentrations reached nearly 500 mM NaCl (Binzel *et al.* 1988). A significant decline in K^+-uptake caused by salinity leads to K^+ deficiency. A K^+-decline could occur when external Na^+ blocks K^+-uptake through some specific transporters.

A large number of genes encode proteins involved in K^+ transport in plants (for review, see Maser *et al.* 2001; Shabala 2003; Very and Sentenac 2002, also refer to Figure 6.1. p 124). These can be divided in two major groups (Shabala 2003):

(1) K^+ channels: At least 4 groups may be distinguished: Shaker-type; KCO; cyclic-nucleotide gated; and glutamate receptors.

(2) K^+ transporters: These include HKT (Na^+/K^+ symporter); HUK /KUP (H^+/K^+ symporter), and K^+/H^+ antiporter.

Among these, several Shaker-type of ion channels are known to be voltage-sensitive. Examples include SKOR and GORK (outward rectifiers; both depolarization-activated; Very and Sentenac 2002); KAT and AKT (hypopolarisation-activated inward rectifiers; Maser *et al.* 2001). Each of these may be potential targets for salt stress, both due to changed channel activity originating from NaCl-induced membrane depolarization (Laurie *et al.* 2002) and as a result of direct block of K^+ channel by elevated Na^+. It remains to be

answered which of these K^+ transporters is the most "vulnerable" to salinity and how their activity can be controlled to maintain optimal K^+/Na^+ ratio in the cell cytosol.

Detoxification and antioxidant defence responses

Salt-stress induces production of reactive oxygen species (ROS) (Sairam and Aruna 2004; Hema *et al.* 2003). How salt stress elicits antioxidant responses in salt-tolerant plants is still not understood (Mostowska 1997). Under increased NaCl concentration, the Na^+/Ca^{2+} ratio also increases, which causes a change in membrane integrity and finally an increase in activated oxygen species (Mostowska 1997; Wimmer *et al.* 2003). Reactive molecules are capable of initiating lipid peroxidation, denaturing of proteins and DNA mutations (Hurst *et al.* 2004). Antioxidant production is usually sufficient to suppress biological damage under normal metabolic conditions, but not during environmental stress events (Hideg 1997) such as salinity. Salt stress elicits antioxidant defence mechanisms. The concentration of antioxidants may vary between tolerant and sensitive cultivars, the more tolerant cultivars producing higher levels of antioxidants, for example in citrus (Avsian-Kretchmer *et al.* 1999), rice (Hema *et al.* 2003), Rhodes grass (Luna *et al.* 2002), and cotton (Meloni *et al.* 2003). A well-developed ROS scavenging machinery is vital to overcome salinity-induced oxidative stress in rice (Hema *et al.* 2003) as well as other salt effected crop plants. Genetic improvement in the salt tolerance of crop plants through the over-expresssion of antioxidant compounds for scavenging activated oxygen species is possibly one way of improving salt tolerance (Mahalingam and Fedoroff 2003; Smirnoff 1998).

Oxidative stress is one consequence of salinity that may be responsible for much of the damage. Salinity produced oxidative stress, indicated by an increase in lipid peroxidation, however the tolerant anger plants (*Cydonia oblonga* Mill.) were much less affected than the salt sensitive loquat plants (*Eribotria japonica* Lindl.). This may be due to the fact that anger plants have a higher capacity to scavenge reactive oxygen species (ROS); anger plants are more salt-tolerant, at

least partly owing to the higher antioxidant enzyme levels observed (Hernandez *et al.* 2003).

6. 5. 4. Osmotic Adjustment

Osmotic adjustment involves the accumulation of inorganic salt in the vacuoles and an increased synthesis and accumulation of organic solutes, namely compatible solutes, in the cytoplasm (Bernstein and Kafkafi 2002; Sakamoto and Murata 2002). It is thought that the compatible solutes not only ensure maintenance of plant turgor but also ensure enzyme activity under high inorganic salt levels (Smirnoff *et al.* 1990).

Compatible solutes

One strategy of salt tolerance is the accumulation of compatible solutes in the cytosol, where they function as osmotic adjusters and provide osmoprotection (Yokoi *et al.* 2002; Gorham *et al.* 1985). Compatible solutes comprise a number of substances including simple sugars, sugar alcohols, quaternary amino acid derivatives, such as proline, glycine and betaine and tertiary amines (Yokoi *et al.* 2002) as well as polyols such as mannitol, sorbitol and inositol (Chen and Murata 2002; Shabala and Lew 2002). They are believed to be involved in salt tolerance mechanisms by contributing to osmotic balance and preserving enzyme activity (Greenway and Munns 1980). Furthermore osmoprotectants enhance stress tolerance by scavenging reactive oxygen species, which are implicated in membrane dysfunction under hyperosmotic stress (Bohnert and Jensen 1996).

Shannon (1997) is suggesting that together with other selection criteria, accumulation traits of compatible solutes might be a useful indicator of salt stress tolerance. Compatible solute production and osmotic adjustment have been associated with genetic variation in salt tolerance, but success in exploiting this phenomenon through breeding has been very limited (Noble and Rogers 1992). Earlier it was believed that the major function of compatible solutes was osmoregulation alone (Wyn Jones 1989), but it seems increasingly evident that their role is predominantly regulatory, e. g. to adjust metabolic pathways to saline conditions (Bohnert and Shevelava 1998; Bray 1997; Hasegawa *et al.* 2000) and

to regulate the activity of numerous transporters involved in Na^+ and K^+ uptake and compartmentation required for osmotic adjustment (Bohnert and Shen 1999; Bray 1997). Compatible solutes are not toxic to the plant but are energetically very expensive to produce. Yeo (1998) calculated that for every gram of dry weight, the halophyte *Suaeda maritima* would need to accumulate 800 mg of glycine betaine (about 400 mg of fixed carbon), which in an agricultural context, would constitute a huge yield penalty.

Inorganic ions

Hyperosmotic stress is known to significantly enhance net uptake of inorganic ions into plant cells, which regulates turgor in osmotically stressed root cells. After onset of hyperosmotic stress an increase in uptake of K^+, Cl^-, and Na^+ by root cells can be expected (Munns 1983). Enhanced cellular uptake of inorganic ions and therefore maintenance of cell turgor might be regulated by compatible solutes (Bohnert and Shen 1999). Shabala *et al.* (2000) found that reduction in cell turgor recovered within one hour of stress onset due to K^+ and Cl^- uptake into bean mesophyll cells. There is evidence that inorganic ion uptake regulates turgor in osmotically stressed Arabidopsis epidermal root cells (Shabala and Lew 2002). If indeed compatible solutes are energetically very expensive to synthesize (Yeo 1998) it might be up to the uptake of inorganic ions to maintain normal turgor pressure as a more economical alternative to compatible solutes (Shabala and Lew 2002; Wyn Jones and Pritchard 1989).

6. 6. GENETIC VARIATION IN SALT TOLERANCE - BREEDING FOR SALT TOLERANCE

The effects of salinity on plant growth are well documented and research publications covering salinity effects number several thousand over the last 30 years or so (Ashraf and Harris 2004; Flowers and Yeo 1997). Although the physiological and biochemical processes involved in response to salinity have been studied in much detail, gaps in our knowledge still remain and the overall management of salt in whole plants continues to be poorly understood (Ashraf and Harris 2004; Cheeseman 1988; Davenport and Tester 2000; Flowers 2004;

Mansour *et al.* 2003). There is no single physiological factor determining resistance or sensitivity to salt, rather resistance is based on an interplay of a number of separate processes, whose genetic determination is not yet understood (Flowers and Yeo 1997; Flowers 2004). The problem facing crop improvement for salt tolerance is complex. Despite many attempts at breeding for salt tolerance little success can so far be recorded (Yeo 1998; Flowers 2004); Ashraf and Harris (2004) are not quite so pessimistic and enumerate several examples of improvement of salt tolerance in crop plants in their latest review over the last twenty years or so, however they point out the urgent need to identify and characterise the underlying biochemical indicators – preferably for individual species rather than generalized for all species – which confer salinity tolerance. Possibly the requirements to deal with environmental stresses such as salinity lie at a level of complexity that so far has bewildered plant breeders. Genetic analyses of quantitative traits may indicate manageable numbers of bits of chromosome having major effects upon complex physiological traits (Prioul *et al.* 1997). Stress responses appear mostly to show quantitative inheritance. Quantitative Trait Analysis examines physiologically complex traits, which are governed by rather few quantitative trait loci. This approach offers potentially greater success in plant breeding for salt tolerance than the idea of a large number of scattered genes each with only a small phenotypic effect.

Genetic variation between genotypes was evident in the rate of ion concentrations in the shoots and root of rice. Rice treated with 200 mM NaCl for one month had a 1.5 times increased concentration of Na^+ and Cl^- ions in the sensitive genotype compared to the tolerant one (Flowers *et al.* 2000). The K^+:Na^+ ratio for the tolerant genotype was twice as high as the sensitive one. Rogers *et al.* (1998) found similar results in lucerne, where Na^+ and Cl^- ion accumulation varied between sensitive and tolerant genotypes, also intraspecific variation of tolerance was observed and attributed to lucerne's heterogenous nature. There is evidence in the literature that salt tolerant cultivars of rice accumulate significantly less Na^+ content in leaf tissue compared to salt-sensitive cultivars (Dionisio-Sese and Tobita 2000). Husain *et al.* (2003) also found different levels

(many-fold) of Na^+ accumulation in leaf tissue of durum wheat genotypes with differing levels of tolerance to salt.

Salt-tolerant plants showed enhanced Na^+ and Cl^- exclusion when compared to the unselected cultivars of lucerne suspension cultures (Chaudhary *et al.* 1996). They concluded that the maintenance of a favourable ratio of K^+ and Na^+ maybe as important as their absolute concentration. Salt-tolerant plants were able to maintain lower Na^+/K^+ ratios than unselected plants.

The problem facing plant breeders in the quest for more salt tolerant lines is determining which of the many mechanisms of tolerance operating in plants will ultimately produce a significant increase in tolerance and at the same time maintain economic yields. It is very likely that the improvement of one of the mechanisms will not lead to an improved outcome (Flowers and Yeo 1997; Gorham *et al.* 1985). It is important to integrate the mechanisms operating at all functional levels: intracellular, tissue and organ levels and also consider the fluctuating environmental conditions that prevail in salinity stressed crops (Ashraf and Harris 2004; Gorham *et al.* 1985). Biological approaches to plant breeding for salt tolerance are affected by the numerous salt-tolerance mechanisms found in higher plants. The initial step of producing more salt-tolerant lines must be the clear identification of all tolerance characteristics, which need to be considered in a breeding program.

Flowers (2004) cautions against exaggerated claims of improved salt tolerance and scrutinizes the literature for hard evidence (improved yield). E.g. he points out that experiments, where no transpiration takes place, are hardly suitable to make claims of enhanced tolerance at the whole plant level. These experiments may, however, provide insights into components of salt tolerance. Yeo (1998) points out that many cellular processes regulating cell based salt tolerance have been characterised genetically and that this adds valuable insights in our understanding of salt tolerance regulation but he cautions that deductions made from cellular processes might lead to unrealistic expectations in solving the salinity problem *per se*. He states clearly that the overriding consideration for salinity tolerance of terrestrial plants is the net flux of water due to transpiration

and so resides at a higher level of organization compared to single cell responses. If intervention at the molecular level is to lead to more salt tolerant plants, it is essential to consider this in the context of the whole plant physiology and approaches to plant breeding (Flowers and Yeo 1997). It is important to remember that cell-based processes may well be subordinate to processes at the whole plant level and one cannot easily extrapolate from single cell behaviour to whole plant responses.

6.7. SALT TOLERANCE IN LUCERNE

Lucerne is regarded as moderately sensitive to salt stress (Maas *et al.* 1977; Shannon 1997) based on yield and when compared to a wide array of other agricultural plants. A general stunting of plant growth is observed and as salt concentrations increase above a threshold level both growth rate and ultimate size of plants progressively decrease (Maas *et al.* 1977). Studies of germination performance of alfalfa varieties had identified more tolerant lines (Dobrenz *et al.* 1983), however this indirect selection at germination has not been found to confer significantly higher tolerance in the field (Johnson *et al.* 1991). High shoot dry weights under saline conditions were highly correlated with low shoot Cl^- (Noble and Shannon 1988). Some research has lead to identification of more salt tolerant alfalfa cell suspensions (Winicov 1997), but Flowers (2004) cautions generally that tolerance on a cellular level is not likely a good predictor of salt tolerance at the whole plant level.

Some comparative studies of salt tolerance in lucerne have found genetic variability in two-week old seedlings (Al-Khatib *et al.* 1994). Also, NaCl-selected lines of alfalfa suspension cultures had a considerably smaller increase in Na^+ and Cl^- than the non-selected cultivars (Chaudhary *et al.*1996). Rogers *et al.* (1998) and Rogers (2001) found significant differences among lucerne lines in relative salt tolerance based on yield and ion concentrations (Na^+ and Cl^-) in the shoots. The intraspecific variation that appears to exist within this species should allow more tolerant lines to be selected.

6.8. CONCLUSIONS

There is sufficient variability within existing cultivars/lines to foreshadow the improvement of salt resistance using mass selection of variable material. Large scale screening of germplasm for salinity tolerance in field trials is difficult because of spatial heterogeneity of soil properties and other environmental factors such as seasonal variation of rainfall and temperature (Munns and James 2003). Physiological traits of salt tolerance, such as Na/K selectivity, Na exclusion, leaf osmolarity, photosynthetic performance parameters etc may prove useful as selection criteria for screening for salt tolerance, rather than yield or biomass *per se*. Effective evaluation methods for selection in breeding trials are necessary to ensure progress in the development of more tolerant germplasm. The complexity of salt stress on overall plant performance provides a challenge for the plant breeder to identify suitable physiological traits for selection and breeding purposes.

CHAPTER 7

MULTIPLE TRAITS ASSOCIATED WITH SALT TOLERANCE IN LUCERNE: REVEALING THE UNDERLYING CELLULAR MECHANISMS

7. 1. ABSTRACT

Salinity tolerance is a complex trait inferring the orchestrated regulation of a large number of physiological and biochemical processes at various levels of plant structural organization. It remains to be answered which mechanisms and processes are crucial for salt tolerance in lucerne (*Medicago sativa L.*). In this study salinity effects on plant growth characteristics, pigment and nutrient composition, PSII photochemistry, leaf sap osmolality, changes in anatomical and electrophysiological characteristics of leaf mesophyll, and net ion fluxes in roots of six lucerne genotypes were analyzed. Three different levels of salinity - low (40 mM NaCl), moderate (80 mM) and high (160 mM) - were used. Treatments were applied to either mature (two-month-old) plants or to germinating seedlings for a period of 4 to 5 weeks. Salt treatments affected most measured parameters in all genotypes, but genotypical differences were obvious for only some of them. Overall, the results suggest that different lucerne genotypes employ different mechanisms for salt tolerance. Sodium exclusion appeared to be the mechanism employed by at least one of the tolerant genotypes (Ameristand). This cultivar had the lowest leaf thickness, as well as the lowest concentration of Na^+ in the leaf tissue. On the other hand, genotype L33 was also tolerant on the basis of growth data, had much thicker leaves and almost double the leaf Na^+ concentration compared with Ameristand (1.76% and 0.91% Na^+ concentration respectively at 80 mM NaCl). Both cultivars showed much less depolarisation of leaf membrane potential than either WL516 or L90. I suggest that salt tolerance in lucerne might be achieved by two different mechanisms, i.e. Na^+ exclusion from uptake (e.g. Ameristand), or effective sequestration of Na^+ in leaf vacuoles (e.g. L33). The

implications of the above measurements for screening lucerne germplasm for salt tolerance are discussed.

7. 2. INTRODUCTION

Salt affects many physiological processes at all levels of organization from the whole plant level to the molecular level. It is not surprising therefore that different plant species employ multiple mechanisms to deal with excessive NaCl content in the soil. At the cellular level, some species such as durum wheat exclude up to 97% of Na^+ from uptake (Munns *et al.* 1999). Such exclusion can be achieved by either preventing Na^+ from entering the root epidermis or, more likely, by its active extrusion from the cytosol back to the soil solution (Tester and Davenport 2003). Recent progress in molecular genetics has identified *sos1* genes encoding putative plasma membrane H^+/Na^+ antiporter mediating such extrusion (Wu *et al.* 1996; Zhu *et al.* 1998). Other species however use Na^+ as a cheap osmoticum (Maathuis and Amtmann 1999), assuming it can be safely stored in the vacuole (Gruwel *et al.* 2001; Mansour *et al.* 2003). Transgenic tomato plants, overexpressing tonoplast Na^+/H^+ antiporter, were able to grow at 200 mM level and produce fruit without any noticeable decline in yield (Zhang and Blumwald 2001). At the whole-plant level, numerous strategies are used by different species to deal with excessive Na^+. These include regulation of Na^+ transport to the shoot at the xylem/root parenchyma boundary (Bouraoui *et al.* 2001; Sobrado 2004), recirculation in phloem (Lohaus *et al.* 2000), compartmentation within the shoot (Flowers *et al.* 1997; Gorham *et al.* 1985; Sairam and Aruna 2004) and salt excretion via specialised organs such as salt glands or bladders (Gorham *et al.* 1985; Marcum 1999; Morales *et al.* 2001). For more details, please refer to Chapter 6.

Improvement of current genotypes by further breeding is one way of combating the ever-increasing salinity problems. Genotypic variation of salt tolerance in lucerne provides scope for selection and breeding of more tolerant lines (Al-Khatib *et al.* 1993; Al-Khatib *et al.* 1994; Chaudhary *et al.* 1996; Noble *et al.* 1984; Rogers 2001). Identification of heritable traits is important for

selecting for enhanced salt tolerance. However, salt stress is genetically and physiologically complex, which seriously affects successful breeding for salt tolerance (Flowers and Yeo 1995). Also, lack of effective evaluation methods for salt tolerance screening, hinders this development (Shannon 1997). As plant screening for salinity tolerance in the field is difficult due to the spatial heterogeneity of soil properties and seasonal variation in rainfall (Munns and James 2003), preference should be given to controlled glasshouse environments. Therefore it is important to identify suitable physiological response mechanisms to salinity and determine whether these are suitable screening parameters for evaluating genotypic variation in salt tolerance. However, no information is available in the literature about what mechanisms are employed by lucerne to deal with excessive NaCl levels in the soil. Only few papers discuss genotypic responses in lucerne to salinity (Al-Khatib *et al.* 1993; Al-Khatib *et al.* 1994; Anand *et al.* 2000; Noble *et al.* 1984; Rogers 2001; Rogers *et al.* 1998) and most of these make assessments on the basis of biomass production alone or else assess nutrient concentrations in plant tissue (Rogers *et al.* 1998). Therefore, the main objective of this study was to shed some light on the cellular mechanisms underlying mechanisms of salt tolerance in lucerne. Several potential features were studied.

Due to the complex nature of salt tolerance in most species, a reasonable approach is to assess a combination of physiological traits for screening germplasm for salt tolerance, rather than relying on a single physiological trait. Therefore, several fundamental physiological characteristics were assessed.

1. ***Plant ionic relations.*** As salinity leads to significant ion imbalances in plant tissues (thus affecting all cell metabolism), Na^+ and Cl^- accumulation or degree of exclusion is potentially a useful trait for salinity tolerance (Rogers and Noble 1992) and maybe less subject to environmental influences than growth rate (Munns and James 2003). K^+/Na^+ discrimination may be another useful trait for selection (Asch *et al.* 2000; Aslam *et al.* 2003; Flowers and Hajibagheri 2001; Zeng *et al.* 2003).

2. Osmotic stress, resulting from salinity is manifest by the disruption of chemical homeostasis and ion distribution within the cell (Zhu 2001). Hence, plant ***water relation characteristics*** such as leaf water potential (ψ) (Rivelli *et al.* 2002a) and osmolality (Marcum 1999; Nanakorn *et al.* 2003) are also potential indicators for salt tolerance.

3. The ultimate effect of the environment on plant growth and yield is mediated by photosynthesis. Therefore, plant ***photosynthetic characteristics*** such as leaf pigment composition and chlorophyll fluorescence characteristics might be useful traits for selection of salt tolerant genotypes as suggested by the literature (Belkodja *et al.* 1994; Corney *et al.* 2003; James *et al.* 2002; Smillie and Nott 1982).

4. Finally, ***electrophysiological characteristics*** such as membrane potential or fluxes of Na^+ and K^+ ions in root tissues have been shown to be very sensitive indicators of adverse salinity effects on plant metabolism (Babourina *et al.* 2000; Carden *et al.* 2001; Cuin *et al.* 2003; Skerrett and Tyerman 1994; Tyerman *et al.* 1997). It remained to be answered, to what extent these characteristics are affected in lucerne, and whether they can be used to distinguish between the degree of salt tolerance in various lucerne genotypes.

The overall aim was to identify mechanisms involved in salt tolerance in lucerne and suggest possible avenues for plant improvement through selection and breeding.

7. 3. MATERIALS AND METHODS

7. 3. 1. Plant Material and Salt Treatment

Two major types of experiments were conducted, with salinity treatment applied to either mature (two-month-old) plants or to germinating seedlings.

In a basic experiment, seeds of six genotypes of lucerne were sown in 1.8 L pots in a glasshouse using standard potting mix (70% composted pinebark, 20% coarse sand and 10% sphagnum peat) with Limil at 1.8 kg/m^3, dolomite at 1.8 kg/m^3, Osmocote Plus™ at 6 kg/m^3, and ferrous sulphate at 500 g/m^3. Six lucerne

genotypes were used: two registered cultivars (Ameristand 801S, WL 516) and four Australian breeders' lines (L288, L33 L90, L235; all from SARDI, Adelaide). Of these, Ameristand is marketed as being salt tolerant (http://www.americasalfalfa.com/ameristand_801s.htm; 6/11/2004), whereas WL516 is not marketed as being salt tolerant. The Australian breeders' lines were chosen based on field observation as being differing in their salt tolerance (Dr Trevor Garnett, personal communication). L288 and L33 are regarded as possibly salt tolerant and L90 and L235 as salt sensitive by plant growers. Plants were grown under ambient light in a temperature-controlled glasshouse (20°C ± 9°C).

Ten days after sowing, seedlings were thinned to seven per pot. The plants were drip irrigated using tapwater with increasing duration and increased frequency up to four times a day for up to 2 minutes each time. Plants were grown under these conditions for 60 days (from July to September 2003), when salinity treatment started. The treatments were 0, 40, and 80 mM NaCl for a period of 5 weeks when plants were harvested and processed for nutrient analysis. Salt treatments were applied by a gravity fed dripper system. Four pots per cultivar/line and salt treatment were established.

In another experiment the same six genotypes were directly sown into soil and thinned to 8 plants per pot 10 d after they were sown. Plants were grown in a glasshouse under ambient light conditions, in a temperature range of 20°C ± 7°C. Four pots per cultivar and treatment were established. For salinity treatment, about 7 g NaCl per 1.8 L pot were added (thoroughly mixed) to the potting mix, resulting in a final NaCl concentration in the soil solution of 160 mM NaCl. Non-saline controls were established (four pots per cultivar) alongside the salt treatment. Plants were hand-watered with tap water once or twice daily upon demand to avoid leaching of salt from the soil. Saucers were placed under each pot to retain any leachate. Any accumulated leachate was returned to the pot. Four-week old plants were harvested and shoot biomass determined.

7. 3. 2. Height and Biomass Measurements

At the end of each experiment, plants were harvested and the height of five main stems per pot, shoot and root biomass were recorded, essentially as

described in Chapters 4 and 5. Plant material was dried at 65°C to constant weight, dry weights were determined and leaf tissue was separated from shoot tissue and ground for further nutrient analysis. Relative water content (RWC) was calculated by subtracting dry weight from fresh weight over fresh weight (FW-DW/FW).

7. 3. 3. Leaf Nutrient Analysis

Dried leaf tissue was digested according to (Zarcinis et al. 1987). The method was modified by adding 1.5 ml of HCl once the plant material had been almost fully digested with HNO_3. Leaf tissue was analysed for P, K, Ca, Mg, S, B, Cu Zn, Fe, Mn, and Na using an Inductively Coupled Plasma Optical Emission Spectrometer (ICPOES ARL 3580 B). Lucerne hay standards were analysed as reference material.

7. 3. 4. Chloride Extraction and Analysis

Finely ground leaf material, dried to constant weight, was weighed into 50 ml capacity bulbous digestion tubes (~0.5 g) and 40 ml of deionised water was added. Plant material was brought into suspension and placed into a 35° C shaking water bath (Ratex) for 90 min and agitated continuously. Digestion tubes were stored at 2° C overnight. The supernatant was subsequently filtered through a 50 µm stainless steel mesh and collected in a 50 ml volumetric flask. Plant material on the mesh was rinsed three times with deionized H_2O and the leachate was also collected in the volumetric flask. Potassium nitrate (KNO_3) was added to the volumetric flask (2.527g; final molarity 500 mM) as an ionic strength adjuster, dissolved and then the extract was brought to volume at room temperature with deionised water. Extracts were stored at 2°C overnight. They were brought back to room temperature the next day and then chloride ion activity was measured using chloride electrode (Ionode Chloride Ion Activity Electrode Model SI 10) in conjunction with double junction reference, Model IJ 46; Ionode Pty.Ltd, Tennyson. The millivolt readings were recorded and corresponding molarity of the extract was calculated using the regression previously determined by

measuring potassium chloride (KCl) standards in the expected range of the chloride concentration in the samples and weight adjusted.

7. 3. 5. Leaf Thickness

Experimental pots received extra watering (~100 ml) of the appropriate salt concentration (0, 40, and 80 mM NaCl) at 6 pm the previous night to avoid potential confounding effects from dehydration. Leaf thickness was measured between 6 am and 8 am EST on the following morning. Leaf thickness was measured on the 3rd or 4th oldest leaf, using the mid-leaflet, by holding the electronic digital callipers (OMNI, Japan) about 2-3mm away from and parallel to the midrib and then gently sliding the moveable jaw of the callipers towards the leaf until the two jaws held the leaf firmly in place. A leaf thickness reading was obtained. Leaf thickness of all treatments (0 mM, 40 mM, 80 mM NaCl) was recorded 5 weeks after treatment started. Three readings per pot (12 readings /treatment/genotype) were taken.

7. 3. 6. Leaf Sap Osmolality

Leaf samples were taken predawn (3 am to 5 am EST). Four to 8 leaflets were severed from the 3rd or 4th oldest leaves and placed into Eppendorf™ tubes, firmly sealed and immediately placed on ice. Samples were sealed in a double layer of ziplock bags, transferred to a –80°C cryo freezer (REVCO, Rheem Manufacuring, Asheville N.C.) for 18 days. For measurements, samples were thawed and then centrifuged at 10000 rpm for 6 minutes to release the bulk sap extract. A 10µl aliquot of each sample to be tested was pipetted onto a small, solute-free paper disc, which was inserted into the Vapro® vapour pressure osmometer (Wescor Inc., USA, Model 5520) sample chamber and sealed. The osmometer reading was recorded. Four replicates per cultivar and treatment were analysed in this way.

7. 3. 7. Chlorophyll Extraction

A sub-sample of about 0.1g fresh weight was taken from between 4 and 8 leaflets using the 2nd to 4th oldest trifoliolate leaf after 3 weeks of NaCl treatment.

Chlorophyll *a* and *b* content was determined spectrophotometrically as described in Chapter 4. Four replicates for each of the cultivars and treatments were analysed.

7. 3. 8. Chlorophyll Fluorescence

Chlorophyll fluorescence was measured at a temperature of 18±3 °C with a PAM portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) in conjunction with a 2030-B leaf-clip holder (Heinz Walz GmbH, Germany) with integrated micro-quantum sensor and temperature sensor. All measurements were carried out in the saturation pulse method essentially as described in Chapter 4.

Fluorescence measurements on dark-adapted plants were taken predawn at 2 – 5 am EST after 3 weeks of NaCl treatment. Measurements were made on the 3rd or 4th oldest leaf, taking care to avoid the midrib. One measurement was taken per pot and four pots per cultivar and treatment were used as replicates, (n = 4). Fluorescence parameters Fv' and Fm' at steady state were measured at midday on a dull (low light conditions) day when ambient light was stable. Measuring light was turned on and waited until Fo' stabilized; Fm' was determined by applying a pulse for 800 ms of saturating white light.

7. 3. 9. Leaf Anatomy

Transverse sections were prepared from fresh leaves, sampled before 9 am on the same day and kept turgid by placing petiole in cooled tap water in a cool environment (12°C) until required for sectioning. Sections were obtained from the mid lamina region of the mid leaflet of the 3rd or 4th oldest leaf sampled 4 weeks after beginning of salinity treatment. A 2-3 x 4-5mm leaf segment was cut from the mid lamina region, avoiding the midrib, and immediately placed into water. The segment was mounted vertically in a 10% aqueous solution of corn syrup on the stage of a Reichert sliding freeze microtome (Reichert, Vienna, No 12054). Transverse sections were cut to a width of 40-70 μm. These sections were gently brushed off into water in a watch glass, mounted on light microscope slides with phenol glycerine jelly. Cover slips were sealed with acrylic nail polish. Leaf

sections were examined with a Leica (model: Leitz DM RBE) light microscope and photographed with a Leica (model: DC 300F) digital camera. Digital micrographs were printed as jpg files and assessed to determine cell size of the columnar palisade mesophyll for control and 80 mM salinity treatment in all six genotypes. A stage micrometer (0.01 mm Olympus,Tokyo) provided a size reference. At least 15 cells (up to 30) from several images (at least four) were measured to determine average cell size.

7. 3. 10. Membrane Potential

The electrical potential difference across the plasma membrane of leaf mesophyll cells was measured in the standard way by impaling the cells with a microelectrode filled with 1000 mol m^{-3} KCl (Shabala and Lew 2002). Leaf segments of 3 x 5 mm were cut from the mid lamina region of the mid leaflet of the 3rd or 4th oldest leaf and left floating on experimental solution (0.2 mM KCl, 1 mM NaCl , 0.1 mM $CaCl_2$ and 5 mM sucrose) essentially as described by Shabala and Newman (1999). The cut segment was mounted and transferred into the measuring chamber. A Perspex holder, which provided a gentle bending of the plant tissue was used, allowing a clear view for electrode positioning compared with planar leaf arrangement. Microelectrodes were impaled using the same hydraulically driven, three-dimensional manipulator as for flux measurements (see later in this Chapter). The reference electrode was a chlorided silver wire inserted in a glass microelectrode with a broken tip (~50 μm diameter) containing 1000 mol m^{-3} KCl in 2% agar. As it was situated at least 6 cm from the measured leaf sample, the diffusion of K^+ ions from it to the leaf was negligible. This conclusion was made based on the absence of any drift in K^+ concentration measured near the tissue in steady conditions. Leaf membrane potential was measured in four lucerne genotypes (Ameristand, WL516, L33 and L90), for controls and for those plants treated with 80 mM NaCl for 4 weeks. In another series of experiments, 80 mM NaCl was added directly to measuring solution a few minutes after the impalement, and resultant depolarisation of plasma membrane potential was measured 20-30 min later.

7. 3. 11. Fabrication of Ion Selective Microelectrodes

Net fluxes of Na^+ and K^+ were measured using non-invasive microelectrode ion flux measuring (MIFE®) technique (University of Tasmania, Australia). Specific details on microelectrode fabrication and calibration are given elsewhere (Shabala *et al.* 1997; Shabala 2000; Shabala *et al.* 2003). Specific details of the MIFE theory can be found in Newman (2001). Briefly, microelectrodes were pulled from 1.5 mm (external diameter) borosilicate blanks (Harvard Apparatus Ltd. Edenbridge, UK, No 30-0053) on a vertical pipette puller and silanized with tributylchlorosilane (Fluka catalog No 90796). Electrode with tips of 2-3 μm diameter were backfilled with 0.5 M NaCl (for Na^+) and 0.2 M KCL (for K^+) and then front-filled with their respective resins (Fluka Chemie AG, Switzerland, Buchs; **Na^+**: Fluka catalog No 71178, **K^+**: Fluka catalog No 60031). Electrodes were equilibrated in an appropriate set of standards (0.2 to 1 mM for K^+; 0.5 to 100 mM for Na^+). Electrodes with a response of less than 50 mV/decade and correlation less than 0.999 were discarded.

7. 3. 12 Ion Fluxes

Lucerne seeds were surface sterilized by immersion in 1% NaClO for 15 minutes then washed thoroughly under flowing distilled water and then germinated on moist filter paper (Whatman No2) in Petri dishes in the dark at 25°C. Plants were used for flux measurements after day 5 at which time, the cotyledons had fully emerged and the primary roots had an average length of about 40 mm. Seedlings were taken out of the incubation chamber and mounted in a 4 ml chamber filled with a bath solution (0.2 mM KCL, 0.1 mM $CaCl_2$, 1 mM NaCl) essentially as described by Shabala *et al.* (2003). Support for the root was provided by fine plexiglass partitions and positioned 2 to 3 mm above the base of the chamber.

The ion-selective electrodes were mounted on a multi-manipulator providing three-dimensional positioning. Net ion fluxes were measured in the mature (8 mm from the tip) zone. Electrodes were positioned 50 μm above the root surface, with their tips about 3μm apart. During measurement, electrodes were moved in a square-wave manner between 50 to 90 μm at a frequency of 0.1

Hz by computerised stepper motor as described in Shabala (1997). Ion fluxes were measured in steady conditions for ~ 5 to 20 min to make sure that no oscillations were present. Then 80 mM NaCl was added to the bath solution, and transient ion flux kinetics were measured for another 40-50 min.

7. 3. 13. Data Analysis

For all growth experiments, each treatment had four replicates, which were arranged in a completely randomised design. The factorial arrangement consisted of six genotypes and three treatments (control, 40 mM and 80 mM NaCl). Treatment effects were determined by analysis of variance (ANOVA) and student *t*-test and P = 0.05 or less was used to indicate significance. Electrophysiological data are based on 5 to 7 reps (individual plants) for each treatment.

7. 4. RESULTS

7. 4. 1. Biomass and Relative Water Content in Plant Tissues

Salinity treatment significantly (P = 0.05) affected both fresh (FW) and dry (DW) weight of lucerne shoots (Fig. 7. 1) and roots (Fig. 7. 2). Biomass decreased with increasing salinity concentrations; normalized shoot dry weight (SDW) at 80 mM NaCl treatment was about 50% of control across all cultivars and root dry weight (RDW) of 80 mM NaCl treated plants was about 25% of control. The effect was stronger on roots, resulting in a significant increase in shoot/root ratio in all except L90 (Fig. 7. 3). Relative water content (RWC) in both shoots (except for L33) (Fig. 7. 4A) and roots (Fig. 7. 4B) increased with increasing salinity treatment. Despite pronounced effects of salinity on the above characteristics, there were, however, no genotypic differences in either biomass (expressed in terms of FW or DW basis) or RWC responses to salinity for either low (40 mM NaCl) or moderate (80 mM NaCl) salinity treatment during 5 weeks. When plants were exposed to a more severe stress (160 mM NaCl) for four weeks, a statistically significant (P = 0.05) genotypical difference was revealed. Three out of six lucerne genotypes (Ameristand, L288, and L33) were

significantly more tolerant, with both salt-treated SFW and SDW values in these plants being twice those of the other three genotypes (WL, L90 and L235).

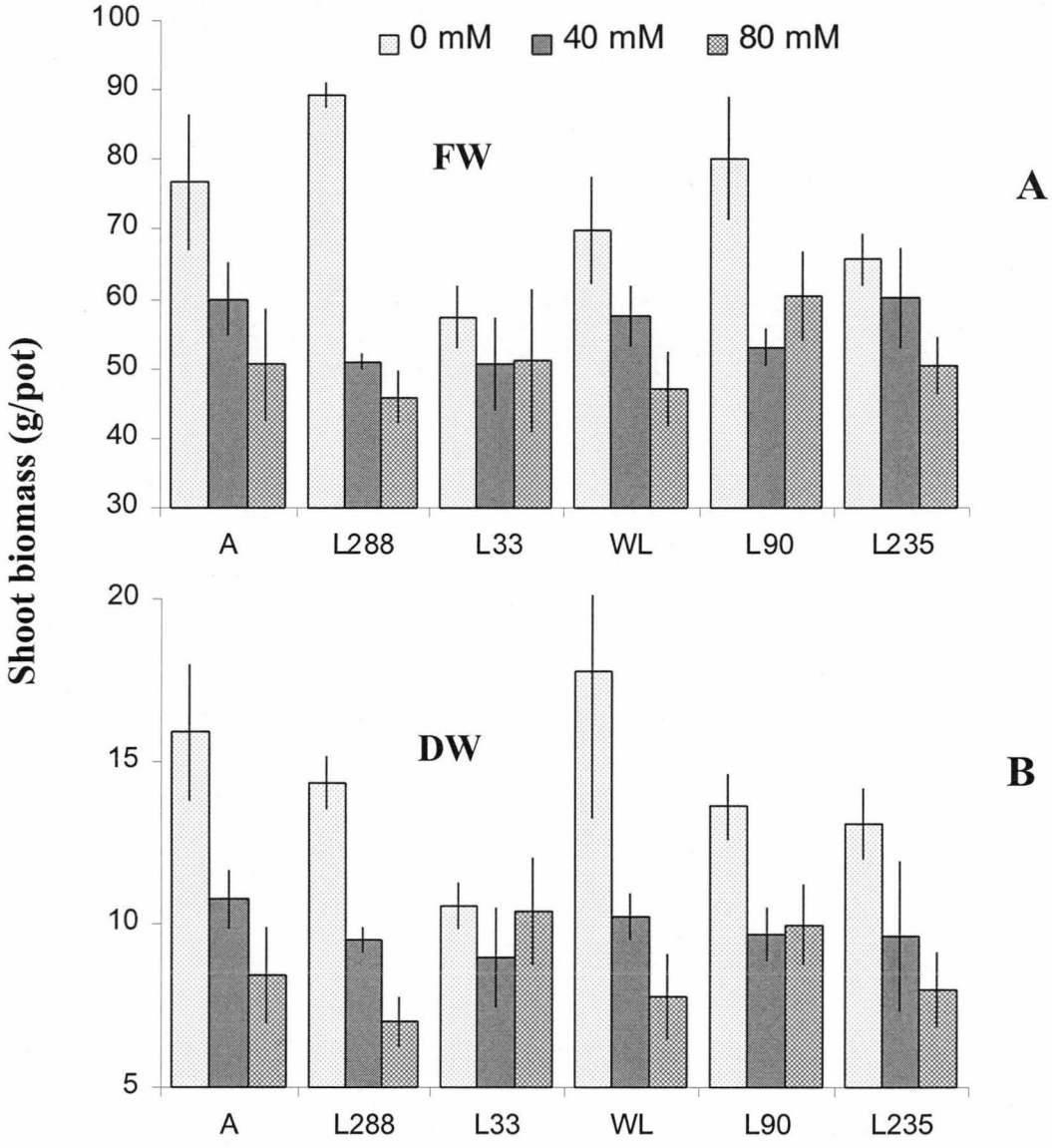

Figure 7. 1. Effect of salinity on shoot fresh (FW; panel A) and dry (DW; panel B) weight of six lucerne cultivars grown at low (40 mM) and moderate (80 mM NaCl) salinity levels. Data are mean ± SE (n = 4).

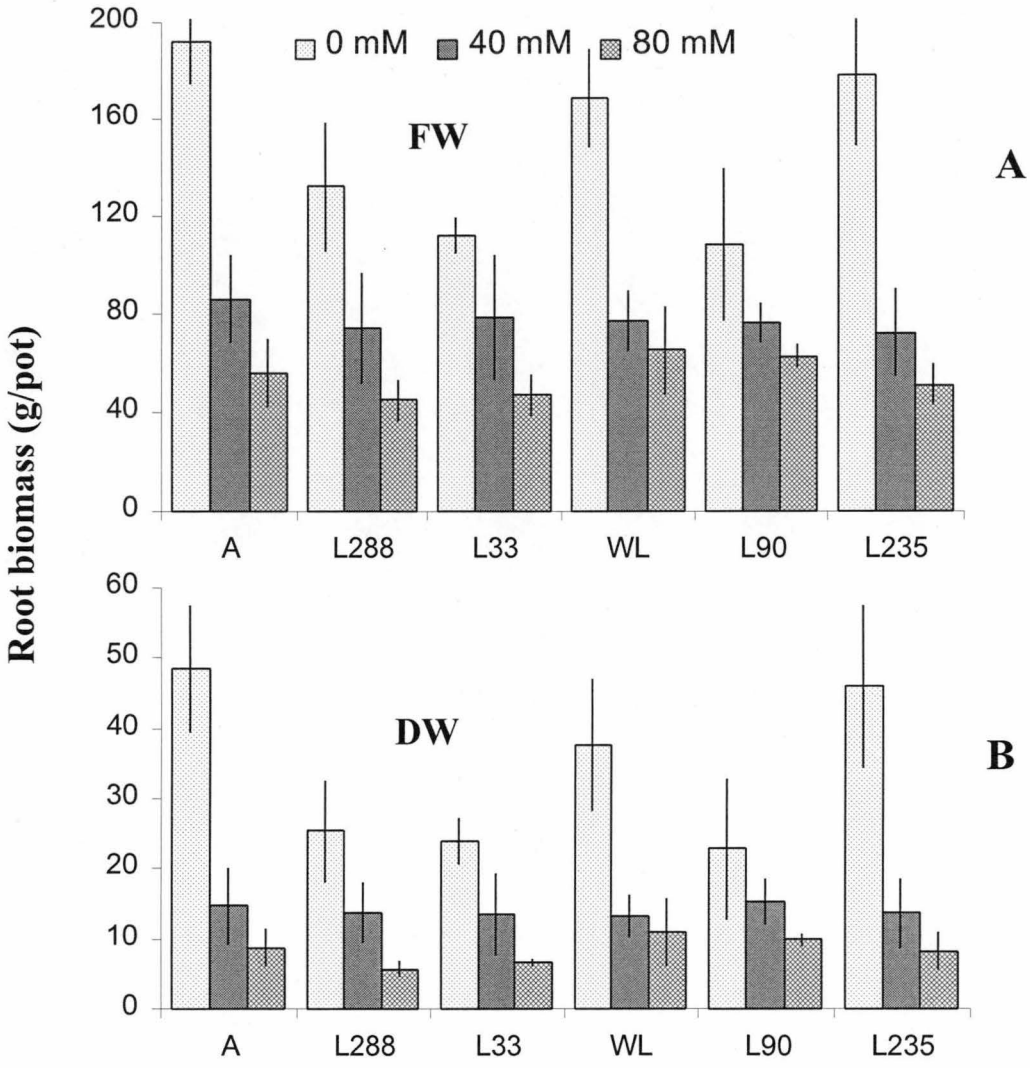

Figure 7. 2. Effect of salinity on root fresh (FW; panel A) and dry (DW; panel B) weight of six lucerne cultivars grown at low (40 mM) and moderate (80 mM NaCl) salinity levels. Data are mean ± SE (n = 4).

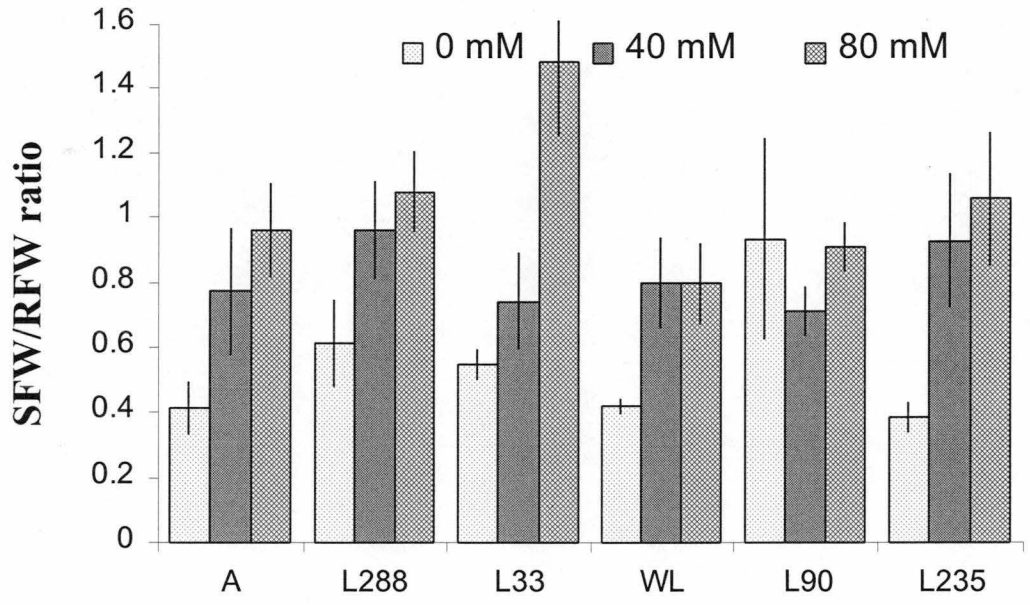

Figure 7. 3. Effect of salinity on shoot/root fresh weight ratio of six lucerne cultivars grown at low (40 mM) and moderate (80 mM NaCl) salinity levels. Data are mean ± SE (n = 4).

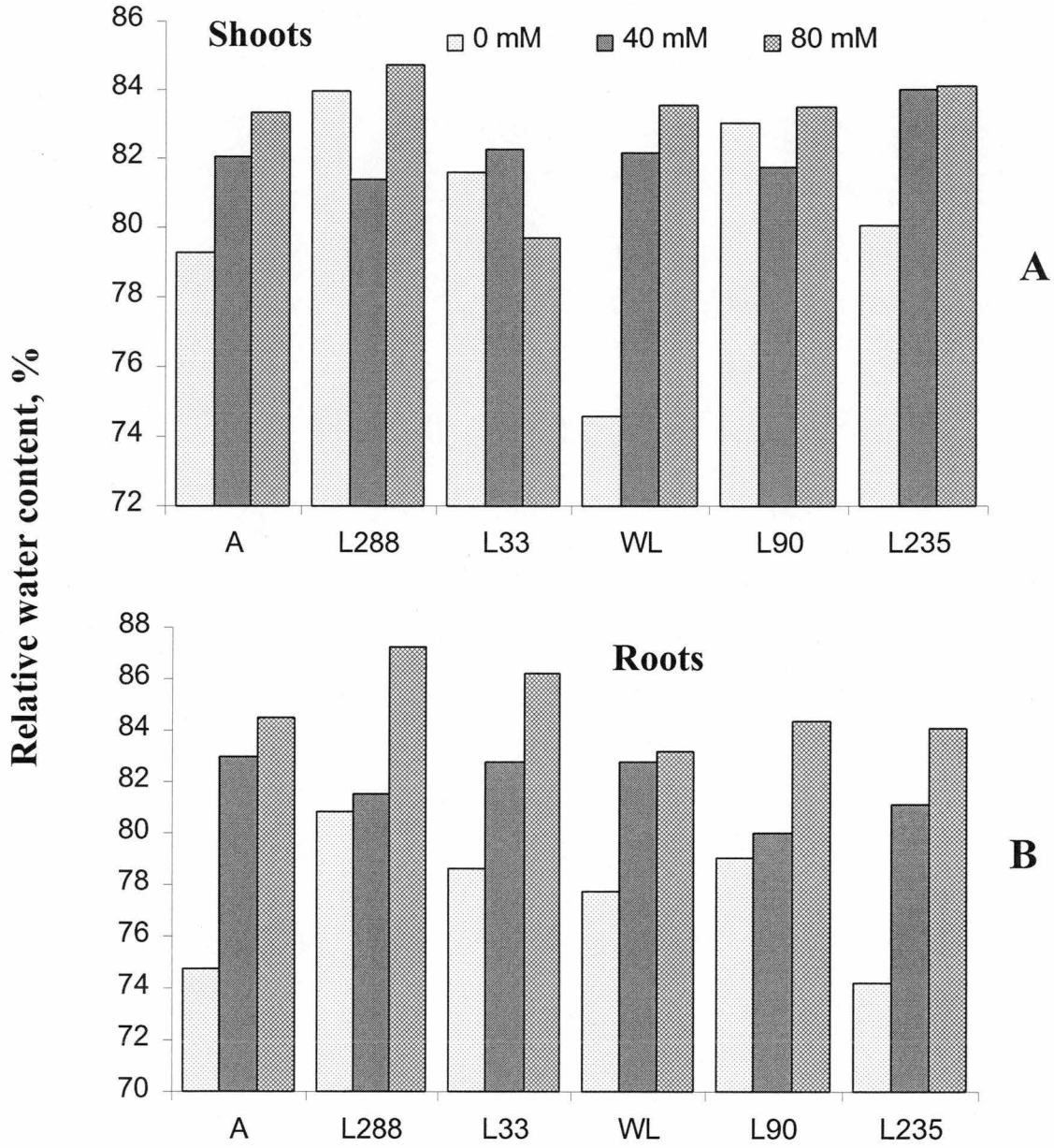

Figure 7. 4. Effect of salinity on relative water content in shoots (A) and roots (B) of six lucerne cultivars grown at low (40 mM) and moderate (80 mM NaCl) salinity levels. Data are mean ± SE (n = 4).

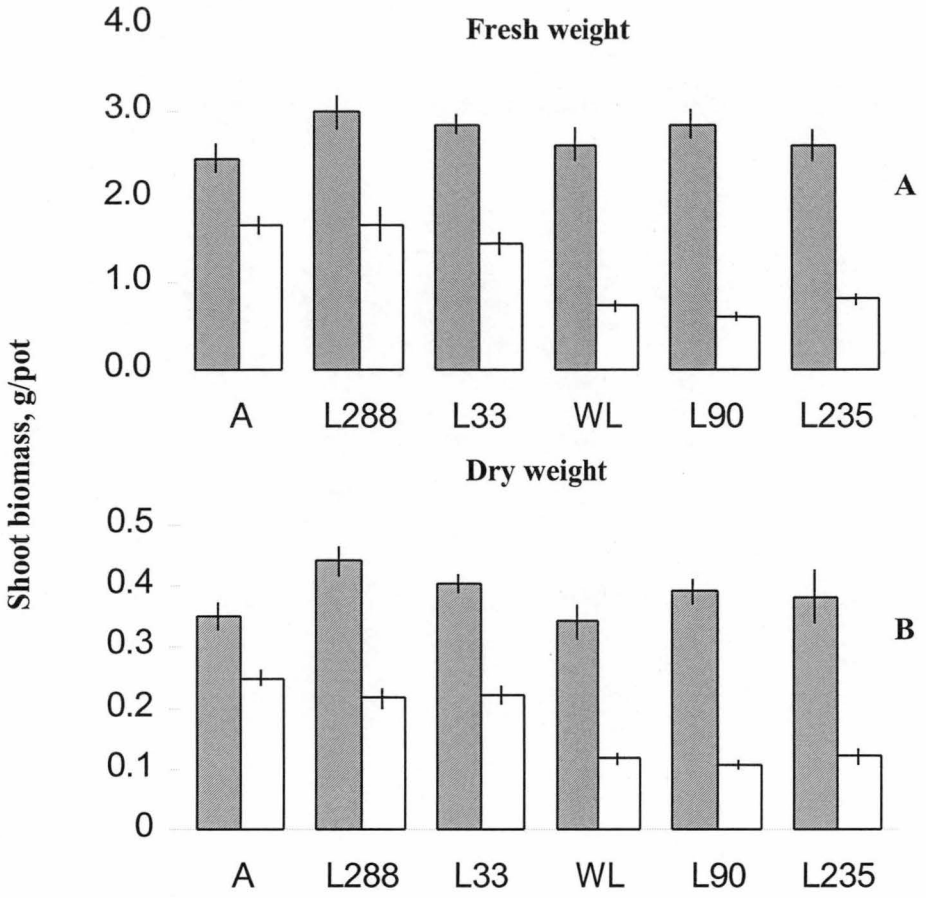

Figure 7. 5. Fresh (A) and dry (B) weight of lucerne shoots after 4 weeks of salinity treatment. Shaded bars – control (no salt); open bars - 160 mM NaCl solution. Data are mean ± SE (n = 4-5).

7. 4. 2. Leaf Photosynthetic Characteristics

Although there was an apparent general trend towards the overall reduction of total chlorophyll content in leaves of salt-treated plants, no obvious correlation with either the severity of salt stress or genotypic differences was found (Fig. 7. 6). In general, Chl b content was more affected by salinity than Chl a. Significant (P = 0.05) reduction in Chl b content between control and salt-treated leaves was found in all but WL516 (due to high SE values) genotypes (Fig. 7. 6B), while Chl a content was not significantly different from control in four out of 6 cultivars (Fig. 7. 6A). No apparent correlation between salt-induced changes in leaf Chl content and plant salt tolerance (see above section) was found.

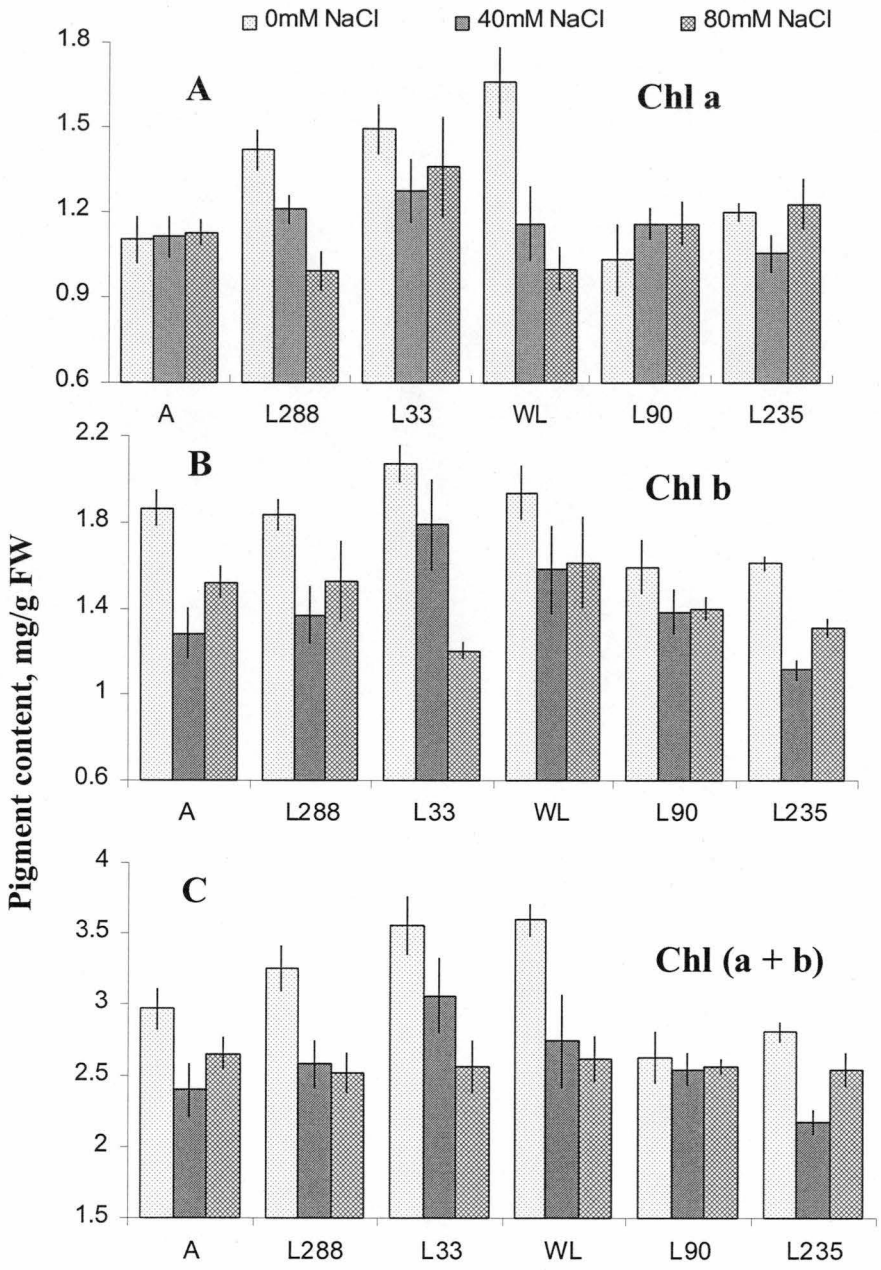

Figure 7. 6. Effect of salinity on Chl a (A), Chl b (B) and total (C) chlorophyll content in lucerne. Plants were grown at low (40 mM NaCl) and moderate (80 mM) salinity levels for 22 days. Data are mean ± SE (n = 4).

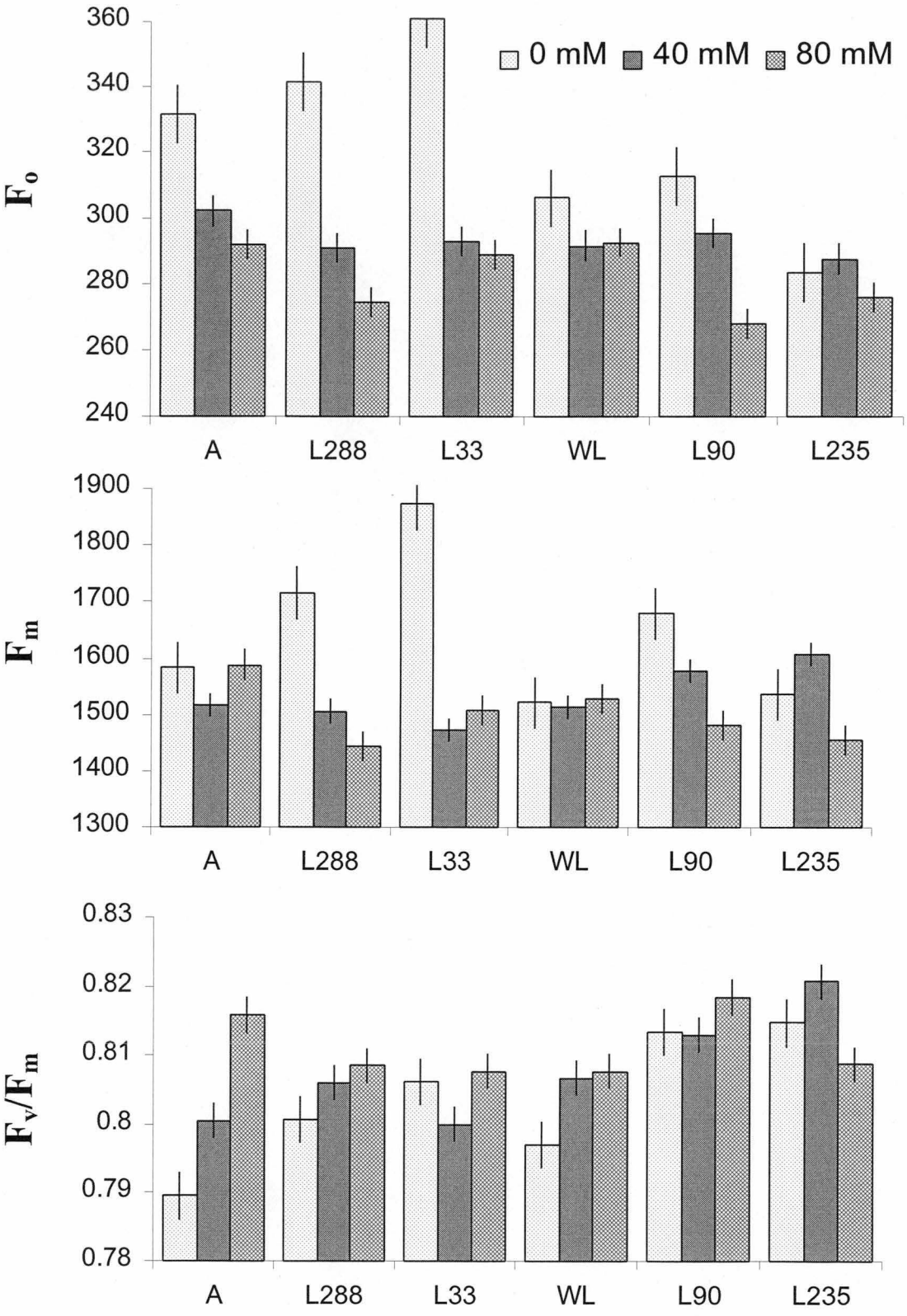

Figure 7. 7. Effect of salinity on chlorophyll fluorescence characteristics of leaves of six lucerne cultivars grown at low (40 mM) and moderate (80 mM NaCl) salinity levels for 24 days. Data are mean ± SE (n = 4).

Leaf photochemical efficiency was estimated by measuring chlorophyll fluorescence characteristics of dark-adapted samples (Fig. 7.7). A significant (P = 0.05) reduction in F_o values was measured in all but L235 lucerne genotypes (Fig. 7.7A). No obvious trends in F_m (maximal fluorescence) changes were observed. As a result, maximum photochemical efficiency in salt-treated leaves was in most cases even slightly higher than in control (Fig. 7.7 C). It could be concluded that no detrimental effects of salinity (40 or 80 mM) on leaf photochemistry were measured in our experiments.

7. 4. 3. Leaf Anatomy

Leaf thickness was significantly (P<0.001) increased in salt-treated plants (Fig.7.8A). This trend was similar in all genotypes, except salt-tolerant Ameristand. In the latter cultivar, leaf thickness at 80 mM NaCl was significantly (P = 0.05) lower than in all other genotypes. The above changes in leaf thickness originated from the substantial increase in the size of leaf mesophyll cells (Fig. 7. 8B; Fig 7. 8C). Again, Ameristand had a significantly smaller cell size at 80 mM NaCl than the other genotypes.

7. 4. 4. Cell Sap Osmolality

Salinity treatment caused a significant (P< 0.001) increase in the osmolality of the bulk leaf sap extract (Fig. 7.9). In 40 mM treated leaves osmolality was on average 60% higher than the control values and 80 mM leaf sap osmolality was almost twice as high as the control values. No obvious genotypic differences were found.

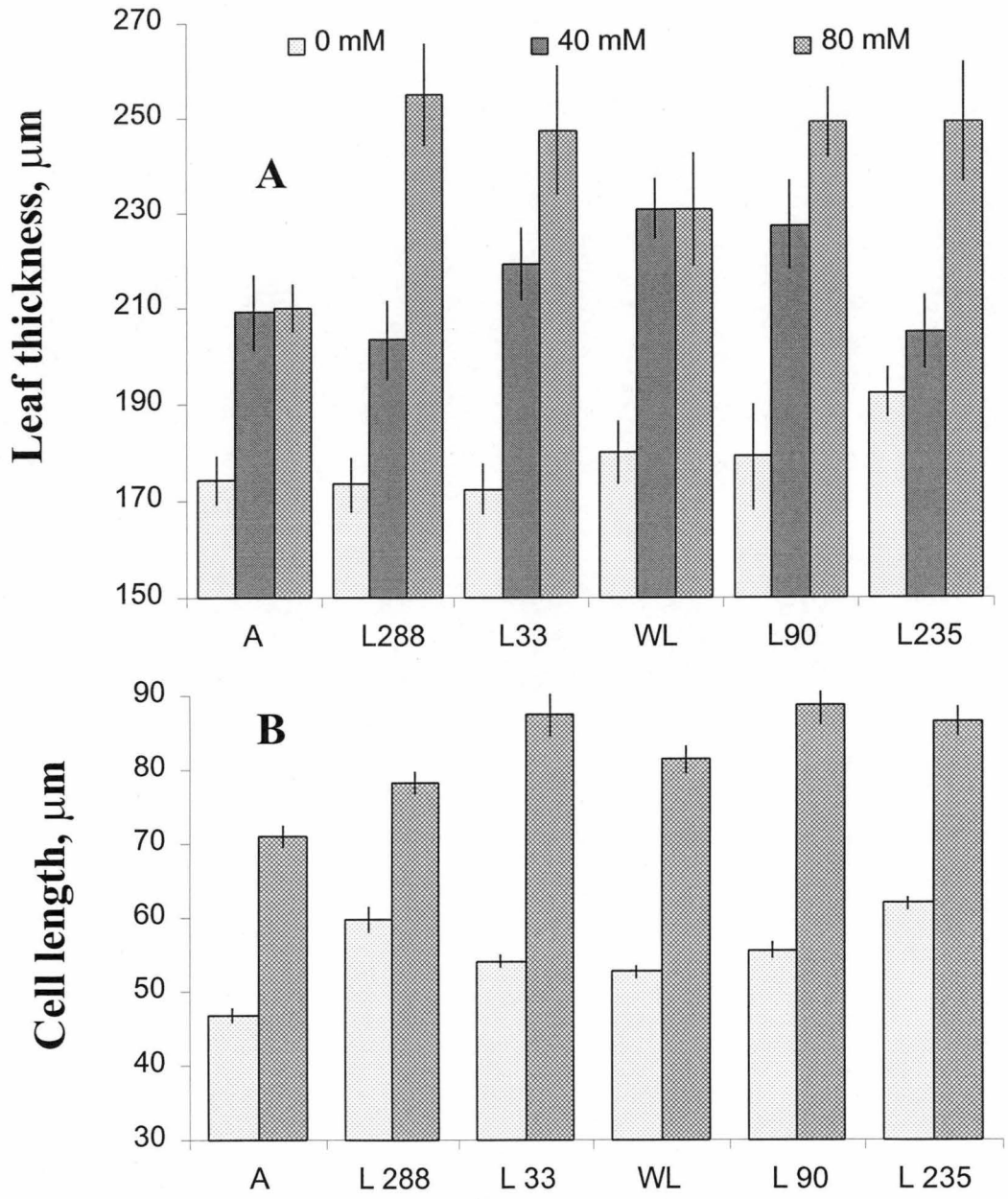

Figure 7. 8. Effect of salinity on the leaf thickness (A) and the length of leaf mesophyll palisade cells (B) in 6 lucerne cultivars grown at low (40 mM) and moderate (80 mM NaCl) salinity levels. Data are mean ± SE (n = 12-30).

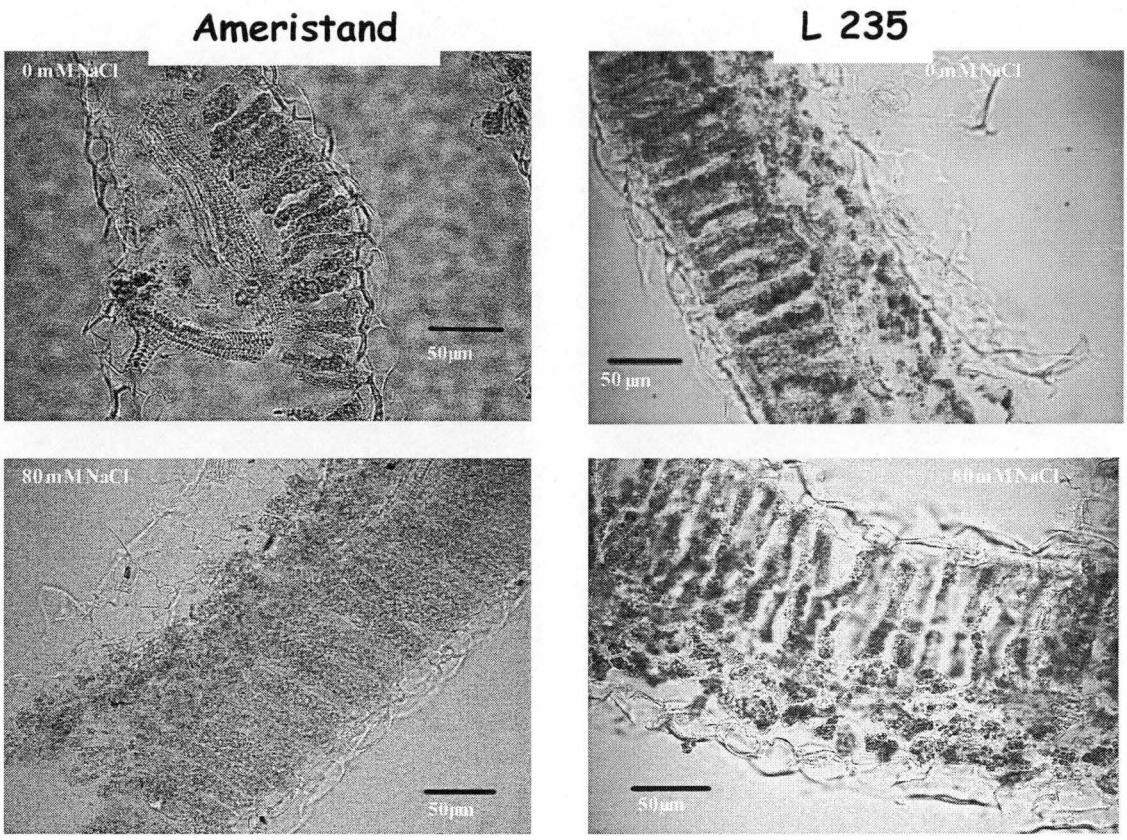

Figure 7.8.C.: Sections of control and 80 mM NaCl treated leaves of Ameristand and L235

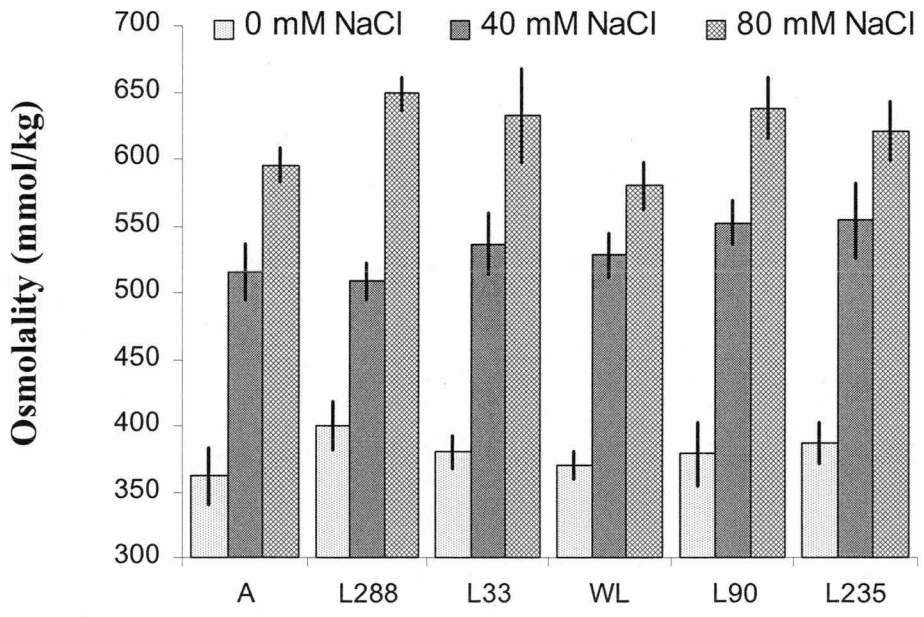

Figure 7. 9. Effect of salinity on the osmolality of leaf sap of six lucerne cultivars. Plants were grown at low (40 mM NaCl) and moderate (80 mM) salinity levels for 22 days. Data are mean ± SE (n = 4).

7. 4. 5. Leaf Nutrients

As expected, salinity caused a significant ($P<0.001$) increase in Na^+ concentrations in plant leaves (Fig. 7. 10A), with an up to ten-fold increase in concentrations between control and 40 mM NaCl treatment and a greater than twenty-fold increase between control and 80 mM NaCl treated leaves. In the 80 mM NaCl treatment Na was lowest in Ameristand, (9.075 g/kg DW of Na in leaves); all other cultivars had substantially higher concentrations. Genotype differences were significant between Ameristand and two other salt-tolerant lines, L288 and L33 ($P = 0.02$ and $P = 0.01$, respectively), as well as between Ameristand and the three salt-sensitive lines. At the lower treatment level of 40 mM NaCl there was no obvious genotypic difference (Fig. 7. 10A).

Potassium concentration of leaves increased (significant at $P=0.01$) in salt-treated leaves (Fig. 7. 10B), with the highest K^+ concentration in tolerant Ameristand and L33 lines. Despite this fact, the K^+/Na^+ ratio dropped dramatically in salt-treated leaves (Fig. 7. 11). Between cultivars, a 10 to 30-fold decrease in K^+/Na^+ ratio was measured (Fig. 7. 11). The lowest reduction was in Ameristand (11-fold), and the highest was surprisingly in L33 (32 fold). However, it appears that L33 had initially a much higher K^+/Na^+ ratio in control (Fig. 7. 11).

There was a significant ($P = 0.001$) difference between salt treatments in leaf chloride content (Fig 7. 12). The 40 mM NaCl treated leaves had about a six-fold increase of Cl in leaves compared to controls, and the 80 mM NaCl treatment had on average of a ten-fold increase of Cl compared to control. At 80 mM Ameristand had a lower Cl concentration than the other five cultivars. This cultivar difference was not evident for the 40 mM NaCl treatment, where all cultivars had an average concentration of about 2.5% Chloride/DW (Fig. 7. 12).

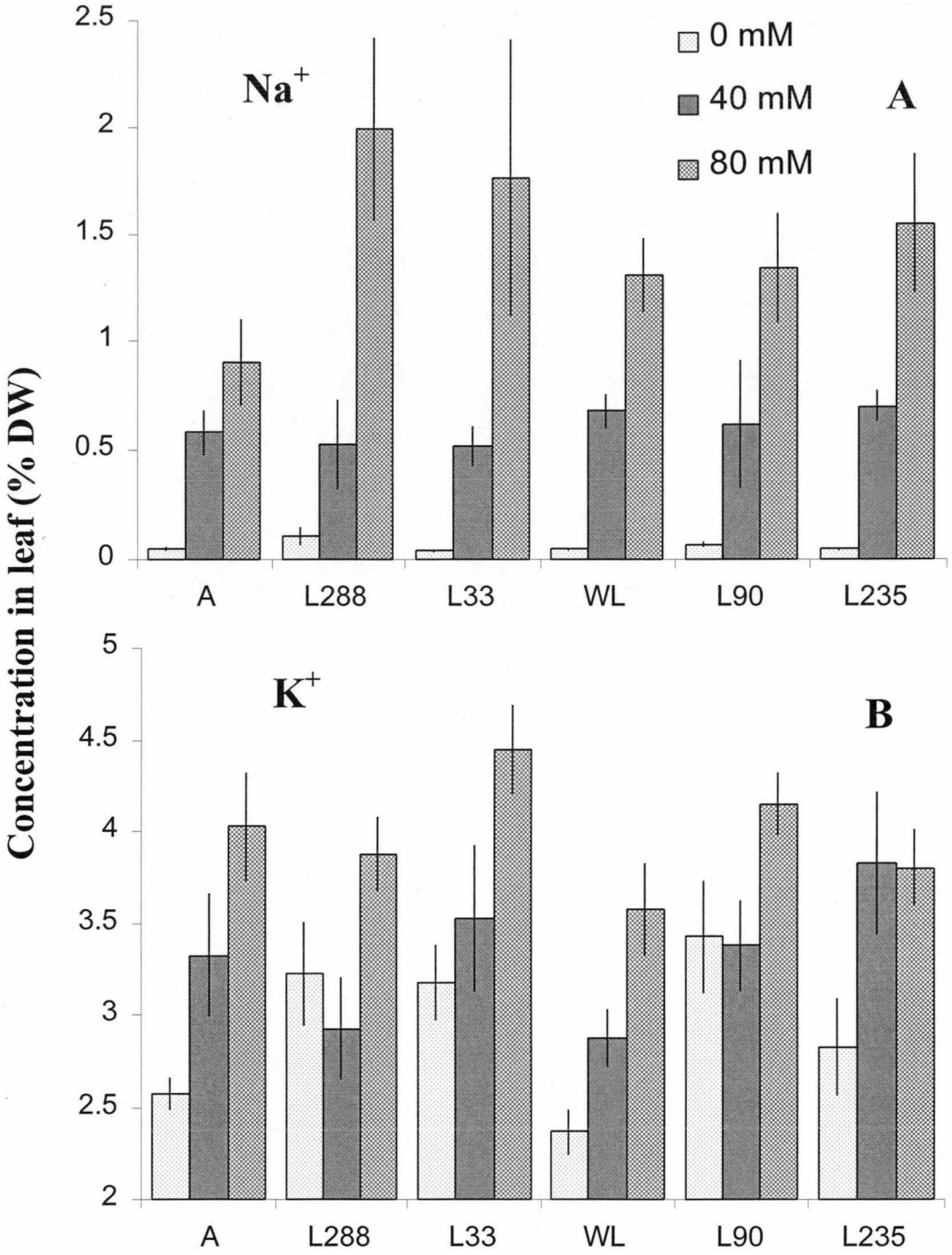

Figure 7. 10. Leaf Na^+ (A) and K^+ (B) content (% DW) of six lucerne cultivars grown at various salinity levels. Data are mean ± SE (n = 4).

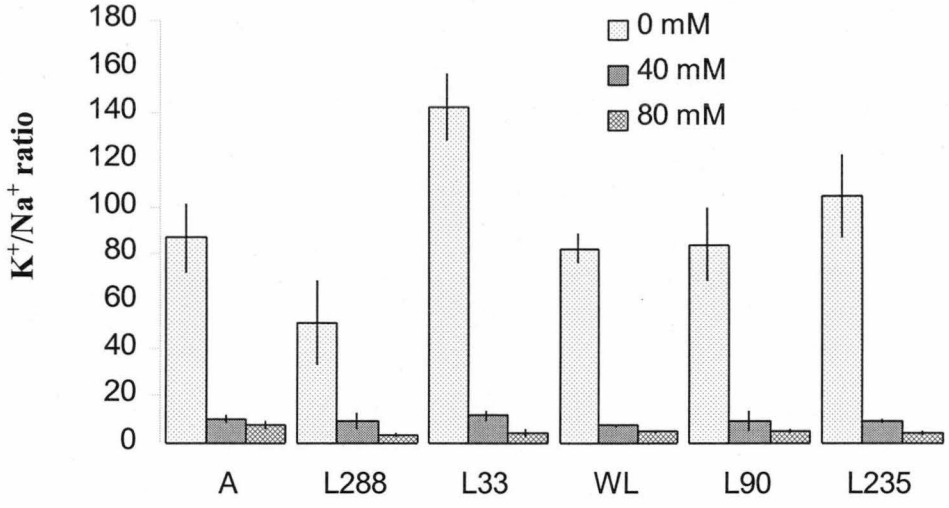

Figure 7. 11. K^+/Na^+ molar ratios in leaves of six lucerne cultivars grown at various NaCl levels. Data are mean ± SE (n = 4).

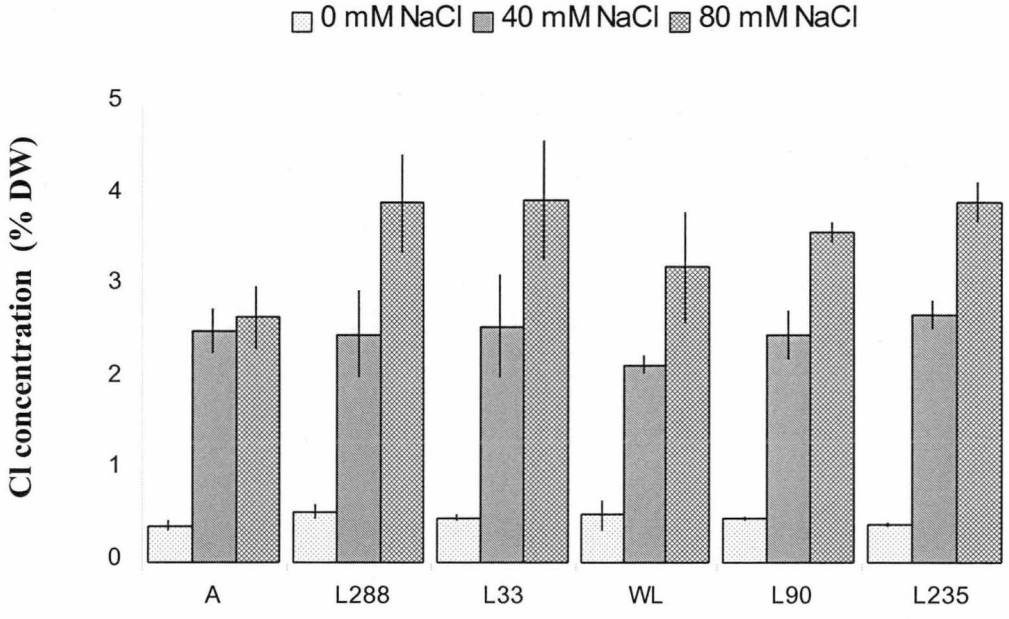

Figure 7. 12. Effect of salinity on chloride content in leaves of six lucerne cultivars grown at low (40 mM NaCl) and moderate (80 mM NaCl) salinity levels. Data are mean ± SE (n = 4).

Among the other nutrients, no obvious trends were found. There were no significant differences for Fe, Mg, Ca, Mn between treatments (data not shown). The average for Fe was 132 mg/kg, for Mn 105 mg/kg, for Ca 2.2% of DW and for Mg 0.41% DW. Phosphorus increased with increasing salt levels ($P<0.001$) and genotype differences were also significant at ($P = 0.06$). S showed a significant increase ($P<0.001$) with increasing salt levels. For sulphur, genotypic differences were significant ($P = 0.05$). Micronutrients (B, Cu, Zn) in the salt treatments were significantly different to control ($P = 0.05$-0.001), generally decreasing with increasing salinity; no genotypic difference was discernible; see Table 7. 1).

Table 7. 1. Effect of salinity (0, 40, and 80 mM NaCl) on nutrient composition in shoots after 36 d of salt stress. Values represent means ± SE (n = 4).

Cultivar	P	S	Zn	Cu	B
	% DW			mg/kg DW	
			0 mM NaCl		
A	0.25 ± 0.01	0.29 ± 0.02	59.25 ± 9.96	7.92 ± 0.92	107.15 ± 15.39
L288	0.33 ± 0.05	0.34 ± 0.03	54.13 ± 1.93	8.08 ± 0.76	115.61 ± 7.24
L33	0.31 ± 0.01	0.31 ± 0.02	48.18 ± 4.59	6.32 ± 0.53	112.80 ± 8.10
WL	0.23 ± 0.01	0.22 ± 0.01	60.61 ± 5.04	7.37 ± 0.19	113.55 ± 3.78
L90	0.36 ± 0.04	0.40 ± 0.01	53.00 ± 5.17	7.31 ± 0.63	103.07± 6.70
L235	0.27 ± 0.03	0.28 ± 0.05	47.41 ± 6.23	6.89 ± 0.52	116.47 ± 6.42
			40 mM NaCl		
A	0.28 ± 0.02	0.34 ± 0.02	46.17 ± 3.70	5.47 ± 0.85	106.94 ± 7.80
L288	0.25 ± 0.02	0.35 ± 0.02	56.43 ± 14.23	5.09 ± 0.80	110.63 ± 2.25
L33	0.32 ± 0.03	0.37 ± 0.03	53.46 ± 1.70	6.21 ± 0.31	111.29 ± 7.17
WL	0.30 ± 0.03	0.35 ± 0.01	50.25 ± 3.17	5.61 ± 0.20	106.77 ± 3.41
L90	0.31 ± 0.03	0.34 ± 0.03	55.31 ± 5.61	6.57 ± 1.13	105.90 ± 6.84
L235	0.38 ± 0.04	0.37 ± 0.05	55.51 ± 6.88	5.7 ± 1.27	104.29 ± 14.65
			80 mM NaCl		
A	0.33 ± 0.03	0.37 ± 0.01	43.14 ± 6.04	5.24 ± 0.77	99.55 ± 1.99
L288	0.37 ± 0.06	0.38 ± 0.02	47.47 ± 7.29	5.06 ± 0.52	83.02 ± 1.86
L33	0.37 ± 0.02	0.35±0.04	39.90±4.30	3.92 ± 0.82	79.51 ± 10.85
WL	0.36 ± 0.03	0.38±0.03	52.31±3.66	5.34 ± 0.45	107.71 ± 1.43
L90	0.35 ± 0.03	0.38±0.01	46.48±2.56	5.45 ± 0.27	96.49 ± 9.02
L235	0.39 ± 0.05	0.37±0.01	46.32±6.59	4.68 ± 0.50	96.19 ± 12.87

7. 4. 6. Electrophysiology

Plasma membrane potential (MP) was measured in leaf mesophyll cells in control and 80 mM NaCl-treated leaves (Fig. 7. 13). In control, no significant difference in the steady-state MP values between genotypes was found (data not shown), with MP being in the range of -110-120 mV (as shown for WL516 and Ameristand cultivars in Fig. 7. 13A). In salt-treated leaves, MP was significantly (P = 0.001) depolarised. The degree of MP depolarisation varied significantly between genotypes. In salt sensitive (as judged on the basis of biomass, Fig. 7. 5) WL516 and L90 varieties, MP values for salt-treated leaves were in the range of -60-70 mV, while in the more tolerant Ameristand and L33 MP values were 10 to 15 mV more negative (Fig. 7. 13B).

Another set of electrophysiological data came from the MIFE experiments. Measuring of Na^+ fluxes was complicated by poor selectivity of Na^+ LIX over K^+ and Ca^{2+} (Knowles and Shabala 2005; Chen *et al.* 2005). As a result, only K^+ fluxes were studied. Net fluxes of K^+ were measured non-invasively from the mature zone of 5-d old lucerne roots in an attempt to correlate ion root nutrient acquisition patterns with salt tolerance features. Similar to a previous report on other species (Shabala 2000; Shabala *et al.* 2003), NaCl treatment caused a significant (P = 0.001) K^+ "leak" (net efflux) from the root (Fig. 7. 15). The magnitude of such efflux ranged between -350 nmol m^{-2} s^{-1} in salt-tolerant Ameristand to as low as -700 nmol m^{-2} s^{-1} in 'salt-sensitive' WL516 after 20 minutes of salt treatment.

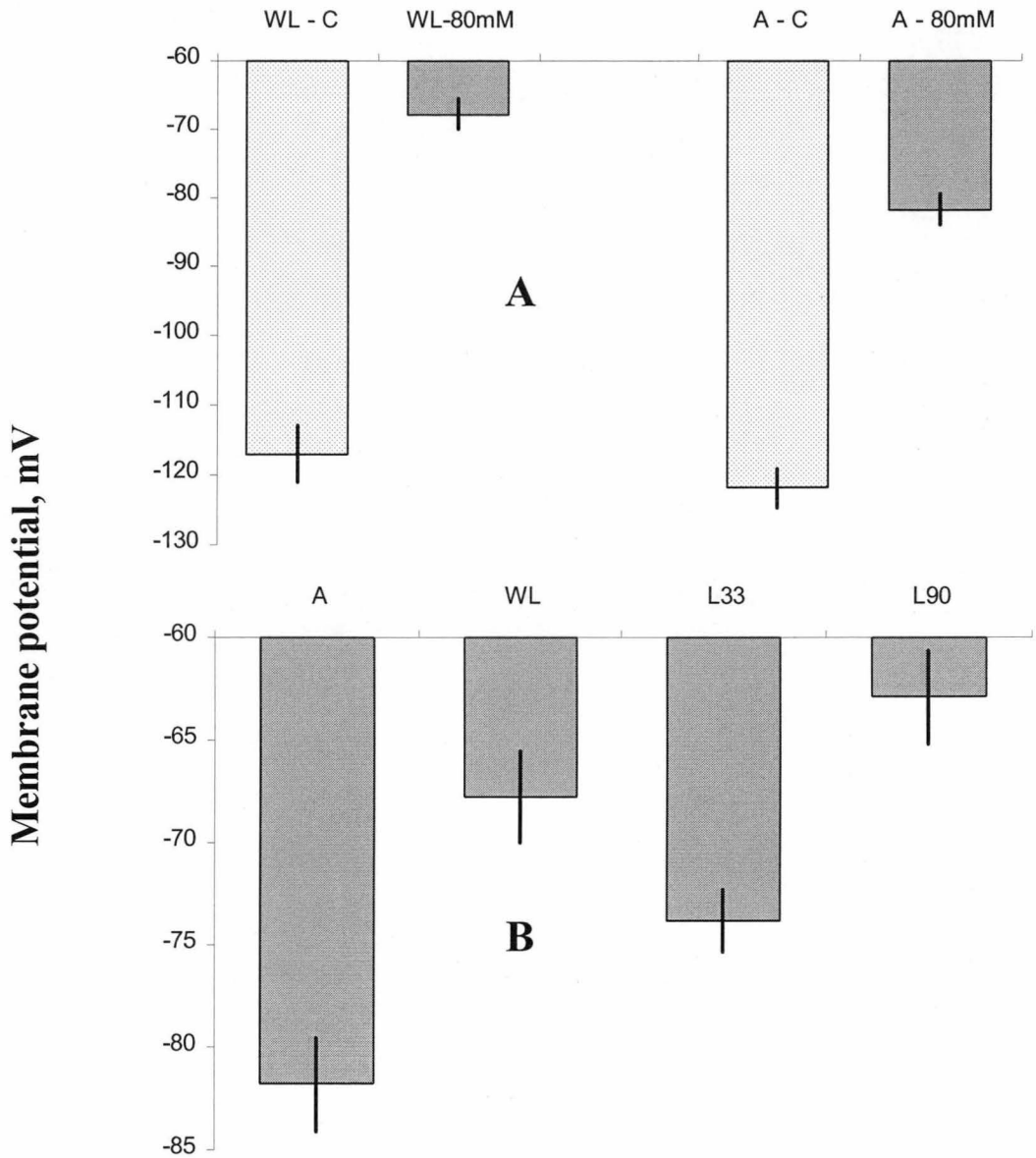

Figure 7. 13. Effect of salinity treatment on membrane potential (MP) of leaf mesophyll cells in lucerne. **A** – steady state MP value before (light bars) and after (closed bars) salt (80 mM NaCl) treatment in two contrasting lucerne cultivars (A – Ameristand; WL – WL516). **B** – steady state MP value in leaf mesophyll cells of 4 lucerne cultivars after 1 h of 80 mM NaCl treatment.

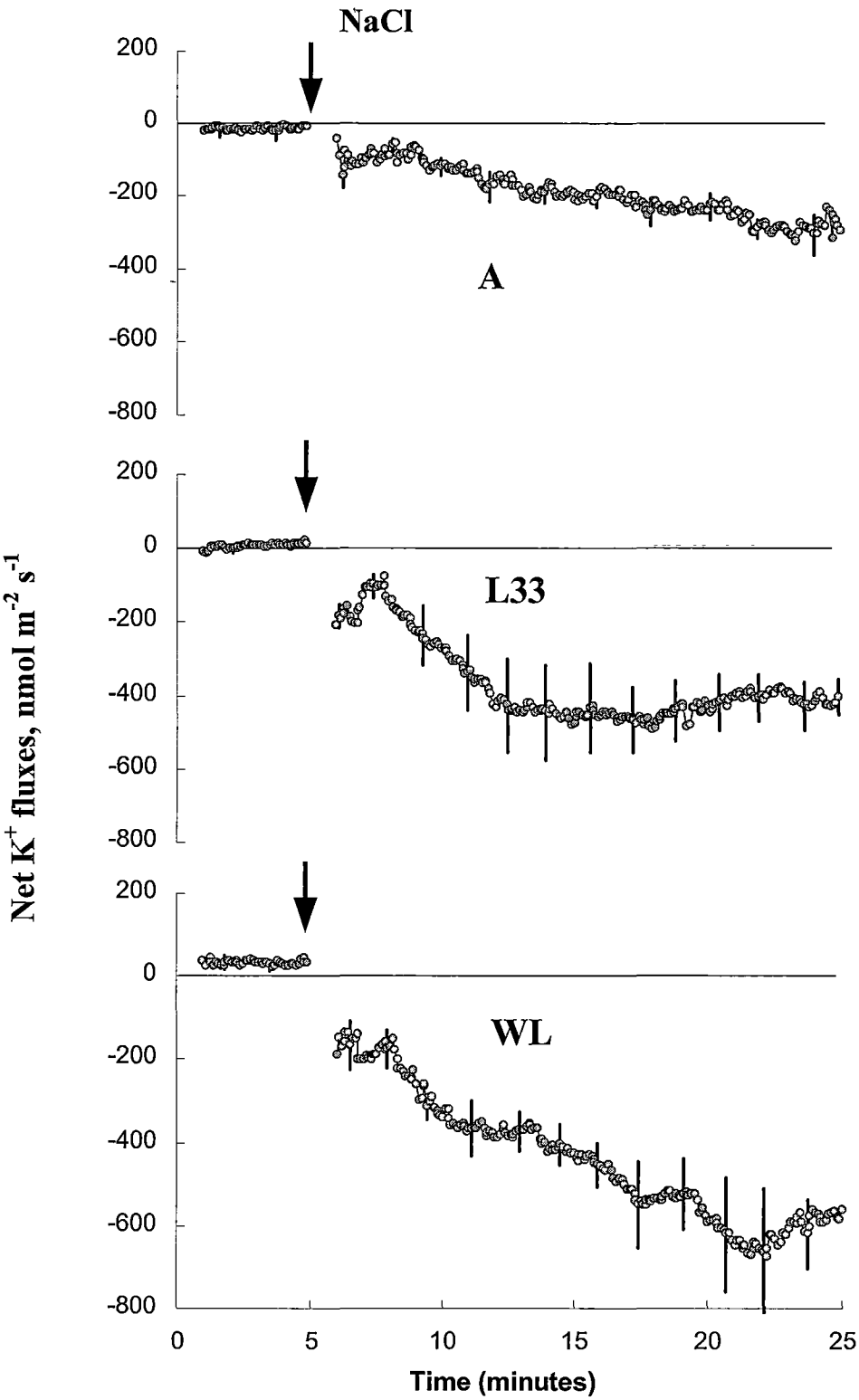

Figure 7. 14. Effect of salinity (80 mM NaCl; indicated with an arrow) on net K^+ fluxes from mature root surface in three lucerne cultivars. Data are mean ± SE (n = 4 to 6).

7. 5. DISCUSSION

7. 5 .1. Biomass

Salinity-induced decrease in leaf and root biomass is a widely reported phenomenon (Alberico and Cramer 1993; Hawkins and Lewis 1993; Rivelli *et al.* 2002b). It comes as no surprise that similar effects were found in lucerne plants in these experiments (Figs 7. 1 and 7. 2).

Both shoot and root growth is affected by salinity (Alberico and Cramer 1993; Bernstein *et al.* 1993; Cramer 1992; Hawkins and Lewis 1993; Kao *et al.* 2001; Lin and Kao 1995; Neumann *et al.* 1994). In these experiments, the effect appears to be stronger on roots, resulting in a significant increase in shoot/root ratio (Fig. 7. 3). At the same time, Nicolas *et al.* (1993) found that root growth was more sensitive to salt than shoot growth.

It appears that the impact of salinity on tissue growth is at least two-fold. Firstly, the growing zone in the root and/or shoot apex becomes significantly shorter (Bernstein *et al.* 1993; Zhong and Läuchli 1993). Secondly, the peak values of longitudinal relative elemental growth rate (REGR) are significantly reduced (Cramer 1992; Kao *et al.* 2001). It also appears that mechanisms of growth inhibition in roots and shoots are quite different. In roots, growth inhibition is due to specific ion and not osmotic effect (Kao *et al.* 2001; Lin and Kao 1995). A possible mechanism for that is NaCl-induced reduction in cell wall extensibility and cell wall hardening (Neumann *et al.* 1994). Nuclear deformation and DNA degradation in root tip cells may also occur (Liu *et al.* 2000). In shoots, short-term growth inhibition is due to water stress; the ionic effects become apparent only after several days of salinisation (Cramer 1992; Munns 2002)

Although plant biomass and height were significantly ($P = 0.05$) reduced by both low (40 mM NaCl) and moderate (80 mM) salinity treatments (Figs 7.1, 7. 2), genotype differences were harder to discern. Earlier Munns and James (2003) suggested that long-term experiments are necessary to detect genotypic differences in the effects of salinity on growth to be confident of obtaining reproducible differences in salinity tolerance between genotypes. This is not likely

to be the problem in this work, where NaCl treatment was given for five consecutive weeks. It also cannot be explained by the fact that salt stress was not severe enough, as a >50% reduction in shoot biomass (Fig. 7. 2) and 75% reduction in root biomass (Fig. 7. 1) was found. More likely, the absence of genotypical difference comes from the complex nature of salt tolerance mechanisms employed by lucerne species and the heterogeneous nature of lucerne populations.

More severe stress (160 mM NaCl) applied to germinating seedlings resulted in a much more prominent differentiation of salt tolerance between lucerne genotypes as indicated by biomass (Fig. 7. 5). After 4 weeks of salt treatment, three genotypes (American cultivar Ameristand and two Australian breeder lines L288 and L33) outperformed three other cultivars (WL516, L90 and L235). Two conclusions are obvious from here. First, the six genotypes used in this study appear to be quite different in their potential to tolerate salt stress. Second, although results in Fig. 7.5 suggest that lucerne genotypes may be reliably differentiated at the basis of growth, the procedure is rather time consuming and thus, far from ideal to be used as a screening tool. Also importantly, the biomass data provides no evidence whatsoever on the mechanisms underlying salt tolerance in lucerne. Therefore, more specific physiological responses have to be analysed.

7. 5. 2. Relative Water Content in Leaves

Salt-treated lucerne plants show a small but significant increase in the relative water content in leaf tissues compared with controls (Fig.7. 4). This is contrary to other reports (Fernandez-Ballester *et al.* 1997; Hawkins and Lewis 1993; Sultana *et al.* 2001). Shoot water relations are believed to be especially sensitive to salt stress. Reduced leaf growth in salt-stressed plants is usually attributed to the inhibited cell elongation caused by leaf water deficit (Munns 2002). This may be partially explained by salinity-induced reduction in root hydraulic conductivity (Azaizeh and Steudle 1991; Evlagon *et al.* 1992; Sohan *et al.* 1999). NaCl-induced decrease in leaf water potential has been reported elsewhere (Kao *et al.* 2001). The fact that RWC was maintained (and even

slightly increased) under salt stress conditions suggests that all lucerne genotypes used have developed some efficient mechanisms to deal with osmotic stress imposed by salinity.

7. 5. 3. Leaf Photochemistry: Prospects for Screening

There appears to be a great deal of scepticism in the literature over whether chlorophyll degradation is the primary cause of photosynthetic degeneration and, therefore, the main biochemical factor of the observed growth reduction under saline conditions (Everard *et al.* 1994). Despite that fact, NaCl-induced decrease in chlorophyll level is widely reported (Abdullah *et al.* 2001; Ali-Dinar *et al.* 1999; Kaya *et al.* 2001; Renault *et al.* 2001) and measurement of leaf pigment concentration have been suggested as a potential screening tool for salt tolerance in some species (Bethke and Drew 1992; Huang *et al.* 1995a; Ma *et al.* 1997). Its applicability to lucerne species remained to be validated. Visual assessment of plants in this study suggested that chlorophyll content appeared the same in both control and salt treated plants at the initial stage. Later, on about d 15 of treatment, the salt treated plants appeared to be a darker green than the controls; this may have been due to reduced cell size and reduced leaf expansion caused by the osmotic effect of the salt treatment and a resulting increase in chloroplast density per unit leaf area (Rivelli *et al.* 2002b). After three weeks of salt stress, when chlorophyll content was quantified, no clear difference was observed (Fig. 7. 6), although in some cultivars (such as WL516, L288 and L33), the overall reduction in total Chl content was significantly ($P=0.05$) lower than in control. As one of these cultivars was classified as salt-sensitive (WL516) - based on biomass data, and the other two - as salt-tolerant (L288 and L33; see Fig. 7. 5), it appears that no correlation can be established between leaf pigment content and salt tolerance in lucerne, at least in the studied range of salinities.

Chlorophyll fluorescence data showed that PSII functionality of salt stressed lucerne (at 40 and 80 mM NaCl) is not compromised. Indeed Fv/Fm tends to increase slightly (not significantly) with increasing salt concentrations (Fig. 7.7). This suggests that all plants were able to maintain the metabolic environment enabling optimal leaf photochemistry. The F_v/F_m values for all

treatments were above 0.8, indicating "healthy and happy" plants (Björkman and Demmig 1987; Bolhar-Nordenkampf *et al.* 1989; Lu and Zhang 2000; Mohammed *et al.* 1995). It has to be mentioned again that, at the same time, shoot growth was reduced by ca 50% (Fig. 7. 2).

7. 5. 4. Cellular Mechanisms of Salt Tolerance in Lucerne

Increased leaf thickness observed in this study (Fig. 7. 8A) is consistent with data in the literature data on increased leaf succulency in many species under saline conditions (Delfine *et al.* 1998; Gulzar *et al.* 2003; Jimenez *et al.* 1997; Sobrado 2004). In this study, this succulence was achieved by the increased size of the palisade mesophyll cells (Fig. 7. 8B). In a typical leaf mesophyll cell, about 70% of the cell volume is comprised of the vacuole (Karley *et al.* 2000). Assuming the cell being of cylindrical shape, a 50% increase in the mesophyll cell length observed in our study (Fig. 7. 8B) will result in a similar (ca 50%) increase in the size of the vacuole, allowing more space for Na^+ compartmentation (Gorham *et al.* 1985; Larcher 1995). This might be crucial for genotypes unable to efficiently exclude Na^+ from being transported to leaves.

It has almost been taken for granted by many authors that an ability of plants to minimise net Na^+ uptake by roots is the main feature conferring salt tolerance (Husain *et al.* 2004; Munns 2002). That could be achieved by either restriction of Na^+ uptake into the root epidermis (Maathuis and Amtmann 1999), or by enhanced activity of a plasma membrane H^+/Na^+ exchanger, contributing to Na^+ removal from the root (Tester and Davenport, 2003). According to some estimates, in some species, roots exclude 98% of the salt in the soil solution, allowing only 2% to be transported in the xylem to the shoots, to prevent Na^+ building up with time in the shoot. However, it is not likely that such exclusion is very effective in the lucerne species studied, except for the Ameristand cultivar. Significant (10 to 20 fold as compared with control) Na^+ accumulation was measured in most genotypes (Fig. 7. 10A). Also consistent was the sap osmolality data (Fig. 7. 9). To fully address the issue of Na^+ inclusion vs exclusion in lucerne plants, measurements of Na^+ concentration in the transpirational stream are needed. Overall, this suggests that most lucerne genotypes prefer to use Na^+ as a

cheap osmoticum and transport it to leaves, decreasing leaf osmotic potential and thus overcoming potential dehydration imposed by the osmotic component of salt stress. Increased leaf succulence is likely to be a mechanism for efficient compartmentation of accumulated Na^+ in leaf vacuole, where no damage to cell metabolism is done (Larcher 1995; Niu *et al.* 1995).

The noticeable exception from this trend was Ameristand. In this cultivar, leaf Na^+ content was the lowest among all cultivars measured (ca 50% of all others at 80 mM NaCl). Even the salt-tolerant cultivars L288 and L33 (as determined by biomass data) (Fig. 7. 5) had double the Na^+ concentration of Ameristand. Also, both mesophyll cell length (Fig. 7. 8B) and overall leaf thickness (Fig. 7. 8A) were the lowest in Ameristand for the 80 mM NaCl treatment. It appears that this particular cultivar, which was bred with the aim of producing a more salt tolerant genotype, proved to efficiently exclude Na^+ from uptake and it appears that is has quite different mechanisms of salt tolerance as compared to L288 and L33, which include Na^+ into the transpiration stream. The fact that leaf sap osmolality of Ameristand plants was not significantly different from others might suggest that this genotype achieves its osmotic adjustment by some other means, such as *de novo* synthesis of compatible solutes (Bohnert and Shen 1999; Bray 1993; Vera-Estrella *et al.* 1999). More studies on this issue are required.

7. 5. 5. Hints from Electrophysiology

On the basis of above findings, it appears that different lucerne genotypes employ two different mechanisms for salt tolerance. Sodium exclusion appeared to be the mechanism employed by Ameristand, while L33 and L288 appear to be Na^+ includers. The salt-sensitive genotypes/lines (WL516, L90 and L235) all seem to be Na^+ includers. Two predictions could be drawn from this: (**i**) ionic homeostasis in the mesophyll cells in tolerant lucerne varieties should be affected to a lesser extent than in salt-sensitive varieties, and (**ii**) regardless of the nature of salt tolerance (e.g. exclusion *vs* inclusion), electrophysiological characteristics of cells in salt tolerant cultivars must be similar. The first prediction was based on the fact that the plant's ability to maintain a cytosolic K^+/Na^+ ratio is central to

salt tolerance (Maathuis and Amtmann 1999). The failure of salt-sensitive lucerne cultivars to either exclude Na^+ from uptake by roots or to efficiently compartmentise it in the vacuole will lead to a dramatic drop in this ratio, thus impairing metabolic processes in leaf mesophyll cells. The second prediction was related to the role of plasma membrane potential as a gating factor for numerous transport systems enabling cytosolic K^+ homeostasis under saline conditions (thus contributing to the maintenance of the optimal K^+/Na^+ ratio). The more depolarised the plasma membrane, the larger is the K^+ leak through depolarisation-activated outward-rectifying K^+ channels (Maathuis and Sanders 1995).

Both predictions were tested in these experiments by measuring leaf membrane potential (Fig. 7. 13). Significantly (10 to 15 mV; $P = 0.05$) higher depolarisation was observed in salt-sensitive WL516 and L90 genotypes in 80 mM NaCl-treated leaves (Fig. 7. 13B). Thus, these data are consistent with the above conclusion that two different mechanisms of salt tolerance operate in lucerne.

Further insights into ionic mechanisms underlying salt tolerance in lucerne were gained from non-invasive MIFE measurements on lucerne roots. Consistent with the idea of Ameristand being a Na^+ excluder, peak Na^+ influx in Ameristand roots was considerably lower compared with the other two varieties tested (L33 and WL516; tolerant and sensitive Na^+ includers, respectively). However, the difference was not significant at $P = 0.05$ level, most likely due to inherent high noise level in measured Na^+ flux. According to the theory, the minimum detectable Na^+ flux at 80 mM NaCl in the bath solution is about 1200 nmol m^{-2} s^{-1} (Ryan *et al.* 1990). Most of this error is coming from the thermal noise in LIX and amplifier. This explains the large variation of Na^+ data (Fig. 7. 14) and questions its applicability to distinguish between contrasting lucerne genotypes.

Potassium is the most abundant inorganic cation in non-halophytes and plays a critical role in charge balancing in the cytosol, activation of crucial enzymatic reactions related to photosynthesis and oxidative metabolism, regulation of cell turgor, stomatal functions, and phloem loading (Kochian and

Lucas 1988; Maathuis *et al.* 1996; Shabala 2003). Although high Na^+ concentrations in leaves may help to maintain leaf turgor, they cannot substitute for adequate K^+ levels in the leaves, mainly due to the role of K^+ in protein synthesis (Chow *et al.* 1990). Maintenance of high cytoplasmic K^+ levels is critical to cell metabolism (Carden *et al.* 2003; Leigh 2001). Plant ability to maintain an optimal K^+/Na^+ ratio has always been considered a key feature of salt tolerance (Maathuis and Amtmann 1999) and has often been proposed as a selection criterion for salinity tolerance (Asch *et al.* 2000).

Upon onset of salinity, significant membrane depolarisation occurs (Hawkins and Lips 1997; Niu *et al.* 1995; Warne *et al.* 1996; Shabala *et al.* 2003;) causing significant K^+ efflux from both leaf (Shabala 2000) and root (Shabala *et al.* 2003) tissues. Preliminary work on barley suggested that the magnitude of such a K^+ "leak" may be used as a quantitative measure of salt tolerance in barley (Z. Chen and S. Shabala, unpublished). The results on lucerne (Fig. 7.15) are consistent with these findings. The difference in the magnitude of NaCl-induced K^+ efflux between salt-tolerant cultivar Ameristand and salt-sensitive WL516 was almost 3-fold (Fig. 7.15). Another salt-tolerant cultivar L33 (Na^+ includer) showed also significantly ($P = 0.05$) smaller K^+ efflux compared with salt-sensitive WL. Therefore, it may be concluded that measuring NaCl-induced K^+ efflux from roots may be a sensitive tool to determine salt tolerance of specific lucerne varieties. Its practical application for screening purposes is jeopardised however by the fact that the MIFE technique is technically demanding, and ion flux measurements are time consuming.

7. 6. CONCLUSION

Genetic improvement of salt tolerance in lucerne would benefit extensive areas both in Australia and world-wide. Because of the genetically complex nature of salt tolerance trait based selection has been recommended for screening (Noble and Rogers 1992). These data suggest that lucerne plants employ at least two very different strategies to cope with salt stress. Salt exclusion from uptake into the shoot (such as in Ameristand) is one of them. The most reliable way of screening

plants employing this strategy are measurements of leaf Na^+ content. The second strategy conferring salt tolerance in lucerne, apparently employed by the majority of lucerne genotypes, is Na^+ uptake in the transpirational stream and its efficient compartmentation in the mesophyll vacuoles. Leaf electrophysiological characteristics (such as membrane potential) may be used as a sensitive indicator of salt tolerance in such plants.

CHAPTER 8

PRELIMINARY STUDIES OF SCREENING FOR SALINITY TOLERANCE USING GERMINATION AND CHLOROPHYLL FLUORESCENCE OF EXCISED LEAVES

8. 1. ABSTRACT

Ten genotypes of lucerne were evaluated for salt tolerance at different stages of plant ontogeny. In one approach, plant screening was based on the evaluation of the germination rate of lucerne seeds. Standard germination techniques were used by germinating seeds in petridishes on filterpaper saturated with NaCl solution at five different concentrations (0, 100, 200, 250, 350 mM NaCl) in an incubation chamber at 20°C degrees. The results showed significant differences between genotypes for germination characteristics under salt stress. For all lucerne genotypes germination percentage decreased significantly ($p = 0.001$) with increasing NaCl treatment. The second technique, potentially suitable as an efficient tool to screen thousands of individual plants, measured chlorophyll fluorescence from excised lucerne leaves, treated with NaCl for a relatively short period (1-2 d) Petioles of excised leaves were immersed in NaCl solutions (0 to 200 mM range). Results showed that maximal photochemical efficiency of PSII (Fv/Fm) decreased with increasing salinity levels. Such decrease was proportional to both duration of salt treatment and severity of salt stress. At higher levels of salinity (150 and 200 mM NaCl) a genotypic response was evident, identifying potentially more tolerant genotypes. The stress of salinity in combination with prior waterlogging resulted in yet a greater reduction in Fv/Fm, indicating an additive response to the dual stress of waterlogging and salinity. Since these two stresses often co-occur it is important to identify genotypes that are tolerant to the combined stresses. The possibility of developing a cultivar better suited to saline and waterlogged soils is discussed.

8. 2. INTRODUCTION

As reviewed in Chapter 6, the capacity of plants to tolerate salinity depends on their ability to exclude salt at the root-shoot interface and the plant's ability to compartmentalize salt in leaves by sequestering NaCl into vacuoles. This mechanism prevents cell dehydration and with it ideally enables physiological functions such as photosynthesis to be maintained (Greenway and Munns 1980).

8. 2. 1. Germination

The range of responses to salinity indicates that it may be possible to use germination trials to select germplasm with increased resistance and identify the more sensitive genotypes as Rumbaugh and Pendery (1990) have pointed out. Germination experiments to identify salt-tolerant genotypes have been undertaken by Ashraf and Waheed (1993); Assadian and Miyamoto (1987); Carlson Jr. *et al.* (1983); Hefny and Dolinski (1999); Rogers *et al.* (1995). Screening germplasm at the germination stage has the added advantage of potentially identifying genotypes at the earliest stage of ontogeny. This screening method is relatively quick, there is no need for specialized equipment, or technical know-how. Thousands of seeds can be screened quickly and it is non-destructive, so individuals that perform well at higher salinity concentrations can potentially be selected and grown on. However early signs of tolerance may not reflect plant behaviour at later stages of ontogeny (Assadian and Miyamoto 1987; Rogers *et al.* 1995; Setter and Waters 2003; Waisel 1991) and these authors were not able to establish a link between germination rate and performance at later stages of ontogeny during salinity. However, Rumbaugh and Pendery (1990) analysed data from over 700 lucerne introduction accessions representing diverse regions of origins and have a convincing amount of data accumulated that suggests that germination screening might identify tolerant germplasm. Therefore I propose that germination rate is potentially one method of identifying salt tolerant germplasm (Ashraf and Waheed 1993; Johnson *et al.* 1992).

8. 2. 2. Chlorophyll Fluorescence

In intact plants PSII is well protected and probably one of the last functional units to suffer from salt stress (Chapter 6 and Chapter 7; Shabala *et al.* 1998).

Therefore using chlorophyll fluorescence screening on intact plants will not reveal stress responses of photosystem II. In the case of excised leaves, however, the root's ability to restrict Na^+ entry and transport to the shoot is eliminated. Therefore, when leaves are immersed with their petioles into NaCl solution, apoplastic Na^+ concentration rises dramatically (especially when leaves are exposed to bright light, which encourages increased transpiration). Na^+ under these conditions will enter the cytosol. This method will potentially identify plants that more efficiently maintain cytosolic Na^+ at low concentrations, either by active extrusion or by sequestering Na^+ into vacuoles and thus be better at protecting PSII. Hence, chlorophyll fluorescence responses might reveal plants that are better able to efficiently exclude Na^+ from the cytosol, especially when considering that of the six genotypes studied (Chapter 7) all but one did not exclude Na from uptake by roots. Therefore excised leaves, as used by Belkodja *et al.* (1999) and Belkodja *et al.* (1994) may elicit a response sooner and at lower salinity concentrations than in whole plants.

The objectives of this study were twofold. Firstly I aimed to investigate whether there exists a correlation between germination rate and photosynthetic performance of excised leaves due to exposure to salinity, secondly I investigated the effects of salinity, and the combined effect of the dual stress of waterlogging and salinity, on the functioning of the photosynthetic apparatus on excised leaves as a quick and non-destructive method for assessing genotypic variation in salt and waterlogging tolerance.

8. 3. MATERIALS AND METHODS

8. 3. 1. Germination Experiment

Ten genotypes were screened for salinity tolerance in a germination experiment. The seeds were germinated in 0, 100, 200, 250 and 350 mM NaCl in a petridish, 50 seeds per dish, lined with two No2 Whatman Filters. 4 ml of solution was added on the first day and subsequently replenished as required (1.5 ml on day 2). Plates were placed into a growth chamber at 20°C on a 12h:12h day/night cycle using fluorescent and incandescent light of 100 μmol quanta $m^{-2}s^{-1}$. Petridishes were placed in a clear, sealed box to avoid excessive evaporation. Germination rate was recorded on d 2, d 4 and d 7. On d 7 an additional parameter namely average primary root

length was measured. Four replicates per each genotype and salinity concentration were assessed.

8. 3. 2. Chlorophyll Fluorescence of Excised Leaves

The same genotypes of *Medicago sativa* used in the germination trial were screened for salinity and the combined stress of waterlogging and salinity using excised leaves. Genotypes were grown in a glasshouse at the Horticultural Research Centre of the School of Agricultural Science at the University of Tasmania, in Hobart, Australia for 50 d essentially under the same conditions described in Chapters 4 and 5; see Fig 8.1. A) Two genotypes were grown at the same time and establishment of the other genotypes followed at two-week intervals, two genotypes at a time. Four replicates per genotype were established; plants were thinned to five plants/pot. As trials were staggered over time (under identical conditions) further statistical analyses of the data was compromised. However, the technique of using excised leaves to determine salt tolerance, is valid and deserves closer investigation.

Forty leaves were excised per genotype of unstressed lucerne plants for subsequent salinity treatment. Leaves were sampled from the third and fourth most recent fully developed position, cut to the same petiole length, and kept in water until being transferred to the salinity treatments. Leaves were placed into 50 ml vials with a small opening in the lid to avoid evaporation and rising salt concentrations in the solution (Fig 8.1 B). Excised leaves were placed into vials containing 0, 50, 100, 150 and 200 mM NaCl solutions and kept for 24 h and 48 h under low light (cold fluorescent) at photon flux of 60 μmol $m^{-}2$ s^{-1} at the leaf level (Fig. 8.1 B). Dark-adapted and steady state chlorophyll fluorescence measurements were taken after 24 h and 48 h exposure to salinity and genotype differences were noted.

The above experiment was repeated after an additional waterlogging period of 14 d using the same genotypes to determine, if the additional stress of waterlogging affected the fluorescence characteristics of the salt-stressed leaves more severely than the salinity stress alone.

Figure 8. 1: (A) Partial view of waterlogging set-up (Chapters 4, 5 and 8); (B) Excised leaves in NaCl solutions under low light.

8. 4. RESULTS AND DISCUSSION

8. 4. 1. Germination

Medicago sativa seed started germinating after 2 to 3 d and control seeds (0 mM NaCl) attained a germination percentage close to 100% after 7 d. With increasing salinity concentrations, germination rate decreased significantly. Most genotypes had a germination rate at or above 90% in the 100 mM NaCl treatment, but there was a significant treatment and cultivar effect at higher salt concentrations (Table 8.1; Fig 8.2; Fig 8.3).

Table 8. 1.: Germination rate of all genotypes in 100, 200 and 250 mM NaCl after 7 days

Cultivar	100 mM NaCl	200 mM NaCl	250 mM NaCl
Sceptre	0.89	0.75	0.48
Super Seven	0.94	0.80	0.42
L90	1.00	0.93	0.57
Salado	1.07	0.89	0.52
L33	0.97	0.89	0.64
SA10070	0.96	0.70	0.21
L235	0.97	0.74	0.27
SA7016	0.86	0.38	0.07
L288	1.00	0.90	0.52
SA35095	0.97	0.87	0.56

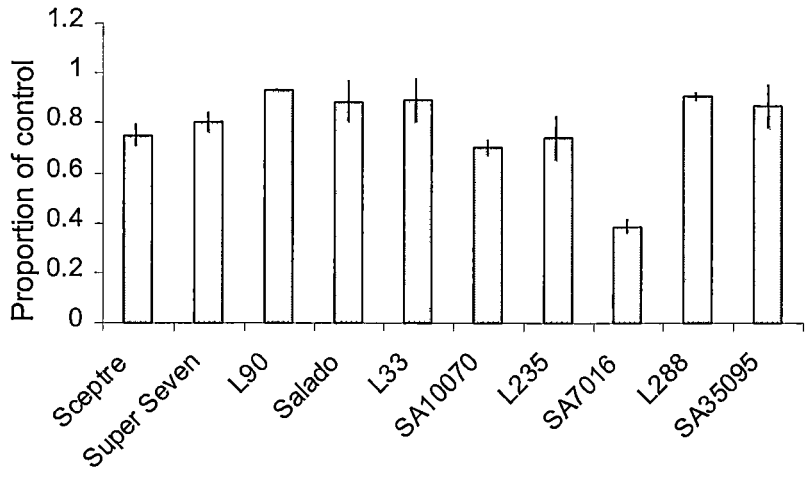

Figure 8. 2.: Normalized germination rate at d 7 of all cultivars in 200 mM NaCl Data are mean of ± SE (n = 4).

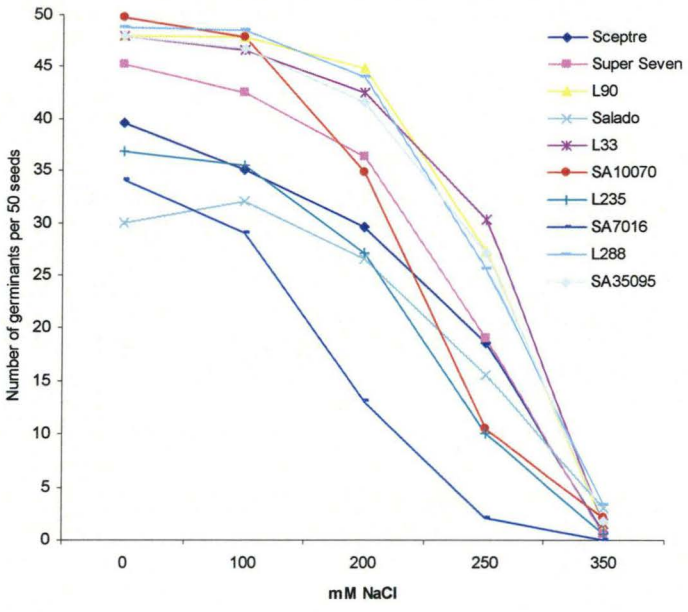

Figure 8. 3.: Number of germinants/50 seeds for all ten cultivars at 100, 200, 250 and 350 mM NaCl after 7d. (For clarity error bars are not included).

Similar responses to salt stress were recorded for *Trifolium repens* in a study conducted by Rogers *et al.* (1995). These authors also noted that with increasing NaCl concentration there were significant differences ($p<0.05$) between populations.

Radicle length of the most tolerant varieties as indicated in Chapter 7 (L288, L33) was less affected by high salinity concentrations than the other cultivars (Hefny and Dolinski 1999). At 200 mM NaCl, L90 and L288 were the only two genotypes to reach 90% germination. SA7016 performed the poorest with a germination rate of 38% at 200 mM NaCl (Table 8.1). At 250 mM NaCl the germination rate dropped to around 50% or lower for all cultivars, L33, L288 and L90 still outperforming the other genotypes (Table 8.1). At 350 mM NaCl the germination rate was negligible.

With increasing salinity, germination was generally delayed and consistent morphological damage was noted, e.g. thinner, contorted, and twisted radicles, incomplete cotyledon emergence, and snapped hypocotyl (Figure 8.4). Radicle length decreased with increasing salinity concentrations, and severity of damage to emerging radicles and cotyledons increased. It was noted that cotyledons decreased in size at the

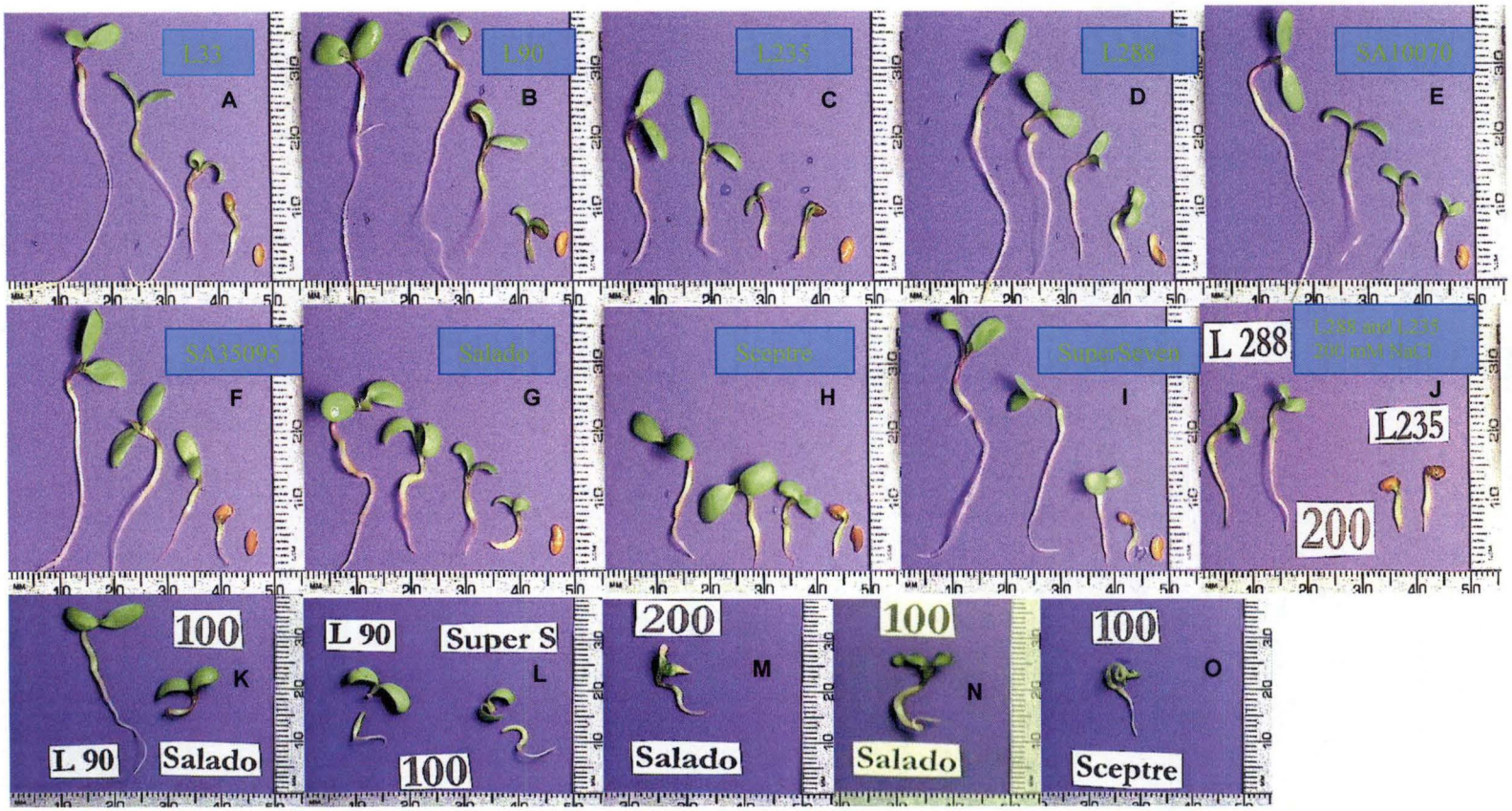

Figure 8. 4.: Representative samples of the germination experiment of nine of the ten genotypes tested at 0, 100, 200, 250 and 350 mM NaCl. Germinants of each genotype were arranged from lowest to highest salinity concentration from left to right (A to I). Also shown are representative samples of tolerant L288 and sensitive L235 at 200 mM NaCl (J), L90 and Salado at 100 mM NaCl (K), snapped hypocotyls of L90 and SuperSeven at 100 mM NaCl (L), severe chlorosis at the tips of cotyledons in Salado at 200 mM NaCl (M), contorted primary roots in Salado at 100 mM NaCl (N), and severe deformation in sensitive Sceptre (O).

higher salt concentrations (250 and 350 mM NaCl) and became dark green, stunted and severely chlorotic at the very tip (Figure 8. 4). At 350 mM NaCl, germination had almost completely ceased, but all germinations at this concentration were incomplete, where cotyledons never fully emerged and both cotyledons and hypocotyl were prone to snapping off. The few individuals that did germinate at this concentration had severe damage to cotyledons and hypocotyl (Figure 8. 4).

The degree of damage appeared to differ between cultivars, but only radicle length of the 200 mM NaCl treatment at d 7 was recorded and analysed (Figure 8.5). There was a significant genotype response in radicle length, and average radicle length of genotypes L288, L33 and L90 was significantly greater than for the sensitive cultivar L235 (Chapter 7).

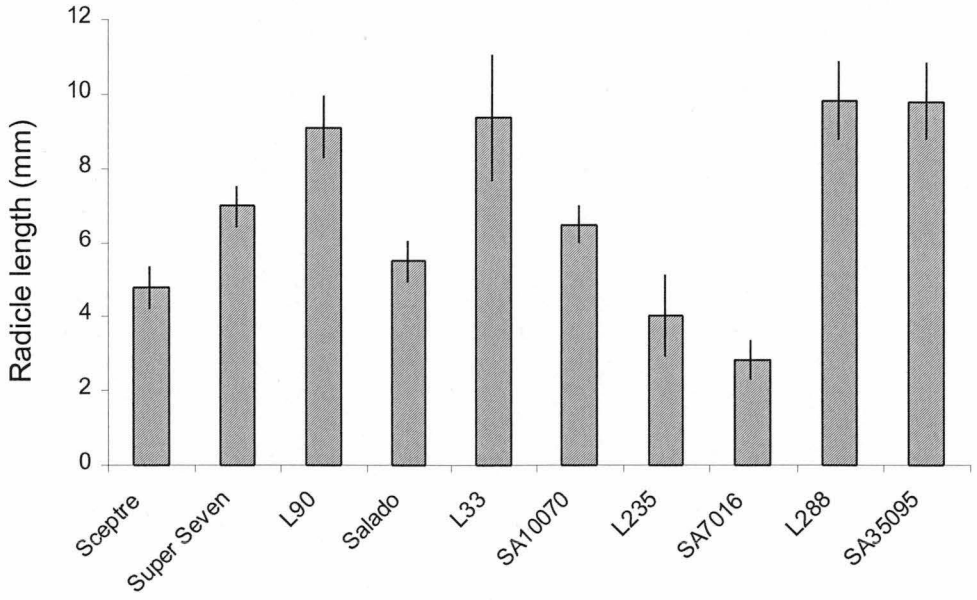

Figure 8. 5.: Average radicle length in mm on d 7 of the 200 mM NaCl treatment. Data are mean ± SE (n = 4). Twelve to 45 radicles were measured/replicate.

Overall there was no strong correlation between Fv/Fm of excised, salt-treated leaves and germination percentage, however several well-performing genotypes (e.g. L288, L33) in the germination trial also performed significantly

better in the chlorophyll fluorescence screening compared to the genotypes identified as sensitive (Chapter 7).

Genotypes that performed well in the germination experiment (e.g. L288, L33) were also identified in another experiment as being tolerant to salt stress on the basis of biomass (Chapter 7). However others (e.g. L90) that performed well during germination were characterized as sensitive (Chapter 7).

It is important however, as Smith and Dobrenz (1987) pointed out, to use seeds of the same quality and age when conducting germination trials. These authors clearly demonstrated that seed age significantly affected performance (at germination) and added a confounding factor to germination results. Cell membrane integrity appeared to deteriorate with increasing age and germination rate declined significantly with increasing seed age. It is a generally held view that germination performance is not well correlated to stress responses at later stages of ontogeny (Rogers *et al.* 1995; Waisel 1991). While this may be so, it was interesting to note in our experiment that some of the well performing genotypes did well not only in the germination experiment but also in experiments with more long-term exposure to the stress of salinity.

8. 4. 2. Chlorophyll Fluorescence

Excised leaves of the control treatment (0 mM NaCl) had values of Fv/Fm ~0.83 (Fig 8.6A). Whereas, at 150 mM NaCl Fv/Fm declined significantly with significant cultivar differences. Chlorophyll fluorescence responses of excised leaves to different levels of salinity clearly showed that salt exposure for 24 h significantly reduced Fv/Fm for all cultivars tested (Fig 8.6B). As expected, with increased exposure (48 h), Fv/Fm decreased even further indicating severe damage of photosystem II of excised leaves (Fig 8.6C). There appeared to be a cultivar/line response to salt stress, however, as trials were staggered over time (under identical conditions) results are not directly comparable. Another experiment, where chlorophyll fluorescence would be measured simultaneously across many cultivars would shed more light on these preliminary results. Chlorophyll fluorescence parameter Fv/Fm decreased even further, when plants had previously been exposed to 14 d of waterlogging stress (Fig 8.7). This is the

first time to my knowledge that the confounding effects of waterlogging and salinity have been analysed in lucerne using chlorophyll fluorescence. There were only two examples in the literature where excised leaves had been used to characterize chlorophyll fluorescence responses in barley (Belkodja *et al.* 1999; Belkodja *et al.* 1994). Belkodja *et al.* (1994) found that changes in rapid fluorescence kinetics due to salinity stress occurred only in the presence of high light, presumably due to a delayed plastoquinone reoxidation in the dark. They pointed out the advantages of using excised leaves to screen for salinity tolerance: genotypes can be germinated and grown under nonsaline conditions and salt being applied to excised leaves only (Belkodja *et al.* 1999; Belkodja *et al.* 1994). This has the potential of speeding up the screening process enormously.

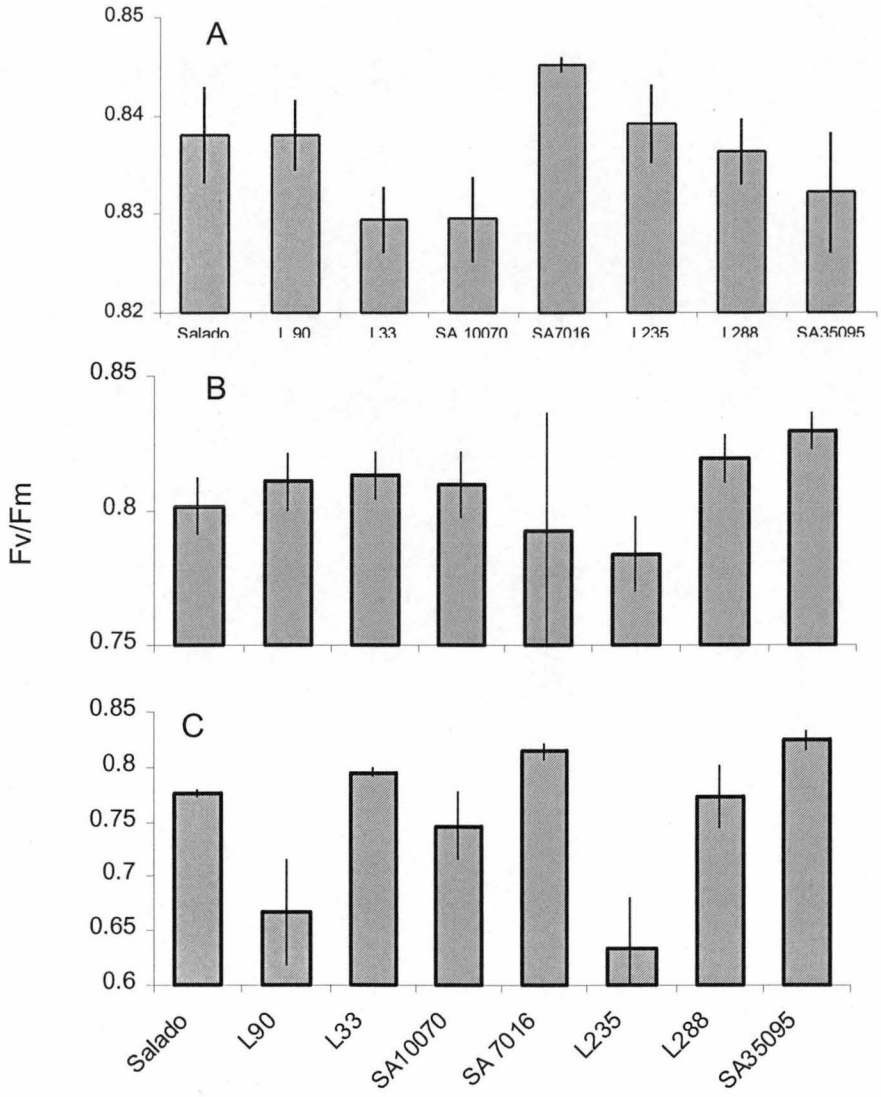

Figure 8. 6. Fv/Fm of control plants (A); Fv/Fm of genotypes exposed to 150 mM NaCl for 24 h (B); and Fv/Fm of genotypes exposed to 150 mM NaCl for 48 h (C). Note differences in scaling of y-axes A, B, and C to allow demonstration of effect. Data are mean of ± SE (n = 5-7).

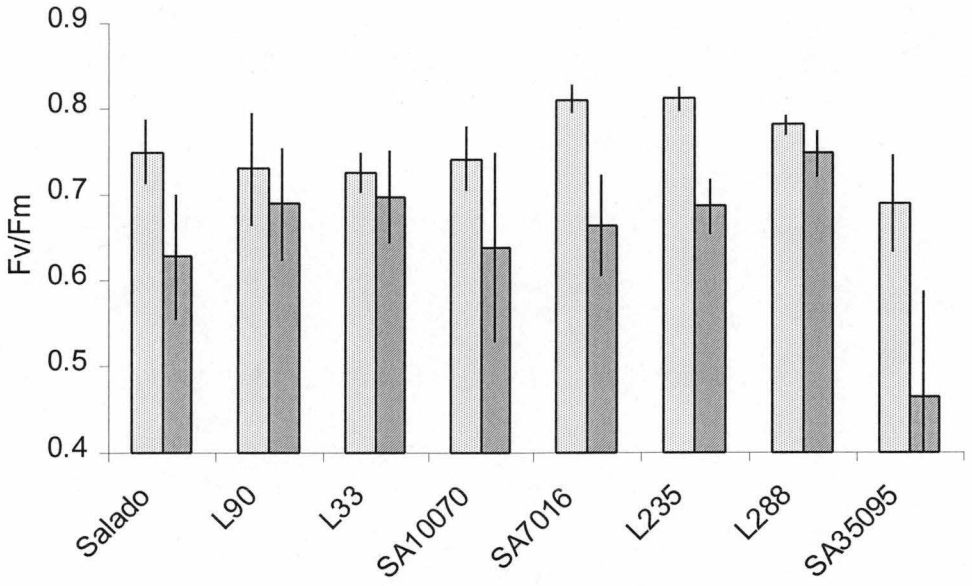

Figure 8. 7. Fv/Fm of excised (previously waterlogged) leaves in 150 mM NaCl after 24 h (light bar) and 48 h (dark bar). Data are mean ± SE (n = 5-7).

L33 and L288, the two breeder's lines that performed well in another long-term salinity experiment (Chapter 7) also performed well here. At the same time, L90 and L235 were poor performers on the basis of fluorescence and also had a much reduced biomass production in response to salt stress compared with L 33 and L288, (Chapter 7).

Results of chlorophyll fluorescence on excised leaves suggest that this method is indeed a useful, reliable, and non-destructive method to estimate the level of Na^+ compartmentation/exclusion and thus allow to make some prediction about the plant's ability to tolerate salt stress. Germination experiments are potentially useful in identifying salt tolerant genotypes and in conjunction with chlorophyll fluorescence may be able to characterize salt tolerance of different lucerne genotypes.

CHAPTER 9

ULTRASTRUCTURAL CHANGES IN LUCERNE LEAVES IN RESPONSE TO SALINITY

9. 1. ABSTRACT

Cell ultrastructural changes due to salt stress were examined in mesophyll cells of two lucerne cultivars. Exposure of two-month-old *Medicago sativa* to 80 mM NaCl for 30 days resulted in a marked disorganization of the thylakoid structure of chloroplasts as revealed by transmission electron microscopy. Thinner granal stacks, relatively large spaces between thylakoid membranes and more prominent plastoglobuli within the chloroplasts were observed in salt-treated plants. These cellular responses are consistent with those reported for other plant species exposed to salt stress.

9. 2. INTRODUCTION

Organelles most affected by salinity appear to be the chloroplasts (Poljakoff-Mayber 1975; Willert and Kramer 1972). Under salt stress, changes in metabolism (Chapter 7) are usually accompanied by changes in the ultrastructural organization of plant cells (Flowers *et al.* 1985; Godde 1999; Khavari-Nejad and Mostofi 1998; Li and Ong 1997; Paramonova *et al.* 2004; Sam *et al.* 2003). A reduction of the thylakoid membrane system has been observed under a variety of stress conditions and the accompanying membrane disintegration might be an explanation for the high number of electron-dense plastoglobuli in chloroplasts of stress-exposed plants (Noble *et al.* 1980; Parameswaran *et al.* 1985). Chloroplasts of plants under stress often have swollen thylakoids with an increased lumen (Wellburn 1994). When *Acrostichum aureum* L., a mangrove fern, was treated with 1% NaCl, disorganization of cellular membranes, structural changes to thylakoids and an increase in plastoglobuli within chloroplasts were observed.

Other researchers reported similar responses in pea (*Pisum sativum* L.) (Hernandez *et al.* 1995) and *Atriplex nummularia* (Niu *et al.* 1996). Structural changes in *Atriplex halimus* leaf cells became more pronounced with duration of exposure and increasing salinity; swelling of grana stacks and intergranal thylakoids progressed to such an extent that margins could not be distinguished and chloroplasts appeared devoid of grana at the highest salt concentration (Blumenthal-Goldschmidt and Poljakoff-Mayber 1968).

Salt stress-induced ultrastructural changes in chloroplasts and thylakoid organization have been examined in halophytes (Kurkova *et al.* 2002; Niu *et al.* 1996), facultative halophytes (Paramonova *et al.* 2004), mangroves (Hwang and Chen 1995; Parida *et al.* 2003), and ferns (Li and Ong 1997) but there are few reports on crop plants (Flowers *et al.* 1985; Hernandez *et al.* 1995; Sam *et al.* 2003) and none on lucerne.

The goal of the present study was to elucidate whether salt treatment would induce ultrastructural changes in mesophyll cells and chloroplasts and whether genotypic differences in the response to salt stress in lucerne mesophyll cells could be discerned. In this experiment, the ultrastructural organization of lucerne mesophyll cells in response to 80 mM NaCl treatment was examined in two cultivars exhibiting varying sensitivity to salt stress.

9. 3. MATERIALS AND METHODS

9. 3. 1. Tissue Preparation

Plant material from the salinity trial described in Chapter 7 was used for the transmission electron microscope (TEM) investigation. Four equal-sized leaves per cultivar and treatment (3rd fully expanded) were excised from two contrasting genotypes, Ameristand (salt tolerant) and WL516 (salt sensitive; see Chapter 7), and two treatments, 0 and 80 mM NaCl after 4 weeks of salt treatment. Leaf tissue hydration was maintained until the samples were ready for fixation. From each leaf, a small tissue sample (3mm × 3mm) was removed from the lamina margin thus avoiding the central vascular tissue. The tissue samples were rinsed briefly in 0.1 M Sörensen's phosphate buffer pH 7.2 (Sörensen 1909),

prior to fixation in 2.5% glutaraldehyde in 0.1 M Sörensen's phosphate buffer pH 7.2 for 15 hr at room temperature. Following two buffer washes (each for 20 min), the samples were dehydrated in an ascending acetone series in 20% increments and taken to 3 changes of 100% dry acetone (each for 30 min), finishing with two changes (each of 20 min) of propylene oxide. The leaf blocks were slowly infiltrated with Spurr's resin of medium hardness (Spurr 1969) and polymerized at 80°C for 24 hr.

9. 3. 2. Transmission Electron Microscopy (TEM)

Polymerised resin blocks were trimmed by hand with a razor blade and mesas cut. Ultra-thin gold sections (0.1 μm thick) were cut with a glass knife fitted to a Reichert Om U2 ultramicrotome. Sections were floated on to 300 mesh copper TEM grids supported with 0.3% Formvar in chloroform (w/v), stained with 1% uranyl acetate in 70% ethanol (w/v) for 10 min and counterstained in lead citrate (Reynolds 1963) for 5 min in order to contrast leaf ultrastructure. Sections were examined in a Philips CM100 TEM at an acceleration voltage of 80 kV. The morphology of cell structures such as the tonoplast, chloroplast and thylakoids between contrasting genotypes and treatments was characterized. TEM images were captured on Ilford plate negatives.

9. 4. RESULTS AND DISCUSSION

This study into the ultrastructural differences between control and NaCl-treated leaf mesophyll cells indicated that some cellular structures undergo morphological changes in response to saline conditions. Chloroplasts within control tissue of both genotypes were distributed 'end-to-end' and more or less continuously around the periphery of the cells (Fig. 9.1). In contrast, the chloroplasts of NaCl-treated cells of both genotypes appeared 'bulbous' and discrete (Fig. 9.2). It has been argued that the rounding of chloroplasts may be due to swelling and that this is the cause of the stroma of chloroplasts to be less dense and the granal thylakoids appearing expanded (Paramonova *et al.* 2004). These cells also displayed extensive but thin peripheral cytoplasmic regions devoid of chloroplasts. Overall, the chloroplasts of control tissue contained fewer starch

granules (Fig. 9.3) compared to the 'bulbous' chloroplasts of NaCl-treated plants (Fig. 9.4). Similar morphological responses in tomato parenchyma cells exposed to increased salt concentrations were reported by Sam *et al.* (2003).

Grana, composed of layered thylakoids, appeared to be much more regular and densely stacked in chloroplasts of control tissue (see Fig 9.5). Furthermore, intergranal thylakoids were distinct and well-developed (Fig. 9.5). In NaCl-treated cells, grana were disrupted and the thylakoids within them were loosely and unevenly stacked. Intergranal thylakoids were difficult to distinguish from the background stroma (Fig. 9.6). Starch granules accumulated in greater numbers in NaCl-treated mesophyll cells (compare Fig 9.3 and 9.4). Changes in thylakoid structure have been reported elsewhere as a typical response to oxidative stress (Bondada and Oosterhuis 1998; Hernandez *et al.* 1995; Salama *et al.* 1994). Maiti *et al.* (2000) observed that many abiotic stresses including salinity caused dilation of the whole chloroplast, separation of grana and accumulation of starch granules in *Phaseolus vulgaris*. Interestingly, Paramonova *et al.* (2004) noted that ultrastructural changes such as the swelling of the granal system were reversible in the absence of other compounding stresses.

Chloroplasts are involved in electron transport and perform phosphorylation (Esau 1977; Jensen and Park 1967). The internal membrane system contains chlorophyll and is the site of the light reactions of photosynthesis. Chloroplasts are one of the first sites of response to diverse environmental stresses, which induce similar symptoms of chloroplast dysfunction and structural damage. Such damage is manifest as starch accumulation, swelling and dilation of thylakoids and plastoglobuli, the destruction of pigments and the consequent inhibition of photosynthesis (Mostowska 1997). The structural alterations might be responsible for reduced efficiency of the photosystem (Parida *et al.* 2003) and reduced electron transport, ultimately resulting in reduced biomass production under salt stress. Parida *et al.* (2003) found a significant decrease in core antenna of PS II of the mangrove *Bruguiera parviflora*, when treated with 400 mM NaCl. This might cause inefficient photon harvesting capacity and therefore lead to reduced efficiency of photosystem II and reduced electron transport activity. The

changes in PS II were accompanied by ultrastructural changes in cells of this mangrove species.

In plants, salinity leads to an increased level of reactive oxygen species (ROS) and, as antioxidant defences are weakened in salt-stressed plants, causes oxidative stress. Although chloroplasts are potentially the most powerful source of oxidants, they are exposed to oxidative stress more so than any other organelle because of their internal O_2 concentrations, especially in the thylakoid membranes. Chloroplasts are therefore particularly prone to generating activated oxygen species (Mahalingam and Fedoroff 2003; Mostowska 1997; Sairam and Aruna 2004). With limited availability of CO_2 to chloroplasts due to stomatal closure, reactive oxygen species build up and in turn can cause peroxidation of chloroplast lipids, damage to thylakoid membranes and pigment breakdown which leads to the reduction or loss of carbon-fixing ability of chloroplasts (Mostowska 1997).

Numerous plastoglobuli were observed in the chloroplasts of salt treated lucerne leaves (Fig 9.6). The functional role of plastoglobuli remains speculative (Lichtenthaler 1968; Tuquet and Newman 1980), however. It is assumed, based on their increase in size and number during thylakoid degradation, that they store thylakoid components, especially those released when thylakoid membranes disintegrate (Lichtenthaler 1968; Lichtenthaler and Weinert 1970; Smith *et al.* 2000). Plastoglobuli may also serve as repositories for surplus lipids (Greenwood *et al.* 1963; Thomson and Platt 1973). Recent evidence suggests that plastoglobuli are exuded through the chloroplast envelope and into the cytoplasm where they undergo degradation (Guiamét *et al.* 1999). In *Atriplex nummularia* L. numerous plastoglobuli were also present in the chloroplasts of salt-stressed leaf mesophyll cells (Niu *et al.* 1996).

The vacuoles of salt-treated mesophyll cells appeared larger than in the controls (See figure 7.8C, Chapter 7) and this may be indicative of excess ion accumulation in the vacuolar space to protect the cytoplasm from toxic levels of ions (Flowers *et al.* 1977; Gorham *et al.* 1985; Huang and Steveninck 1990; Wyn Jones and Gorham 1983). Salinity-induced increased vacuolation has previously

been observed in barley (Huang and Steveninck 1990) and in sweet potato (Mitsuya *et al.* 2000).

Measurements of mesophyll cell dimensions, indicating cell enlargement, may be indicative of vacuolar expansion due to salinity (Chapter 7), an observation consistent with that recorded in parenchyma of salt-stressed tomato plants (Sam *et al.* 2003). Such changes were unable to be confirmed from the TEM micrographs of salt-stressed lucerne mesophyll cells in this investigation as the method of fixation used may have altered vacuole size (Mollenhauer 1986; Oparka and Hawes 1992). The harsh reagents used are known to cause ion leakage and/or dehydration artefacts during fixation (Lee 1984; Mollenhauer 1988), therefore vacuolar size changes could not be confirmed, but such changes are likely (see Chapter 7; Fig 7.8A and Fig. 7.8B).

The ultrastructural comparison of different NaCl-treated genotypes of tomato and rice have previously been reported by Sam *et al* (2003) and Flowers *et al.* (1985). Both authors were able to identify genotypic differences, which manifest as an increased number of cytoplasmic electron-dense corpuscles in the more tolerant of the two tomato cultivars and in a greater degree of disorganization of grana stacks in the more salt sensitive of the two rice cultivars.

It remains to be answered if such genotypical differences occur in lucerne as well. Our preliminary experiments suggested that this might be the case.

9. 5. CONCLUSION

Salinity-induced structural alterations in thylakoids of lucerne mesophyll cells are presumably due to oxidative stress in chloroplasts. It is not clear whether these alterations are a result of leaf adaptation to stress conditions or simply signify organelle damage due to increased levels of ROS in chloroplasts under saline conditions. Further work is needed to investigate the physiological and metabolic significance of these changes, as well as to whether such ultrastructural changes could be used as possible salinity tolerance indicators in lucerne.

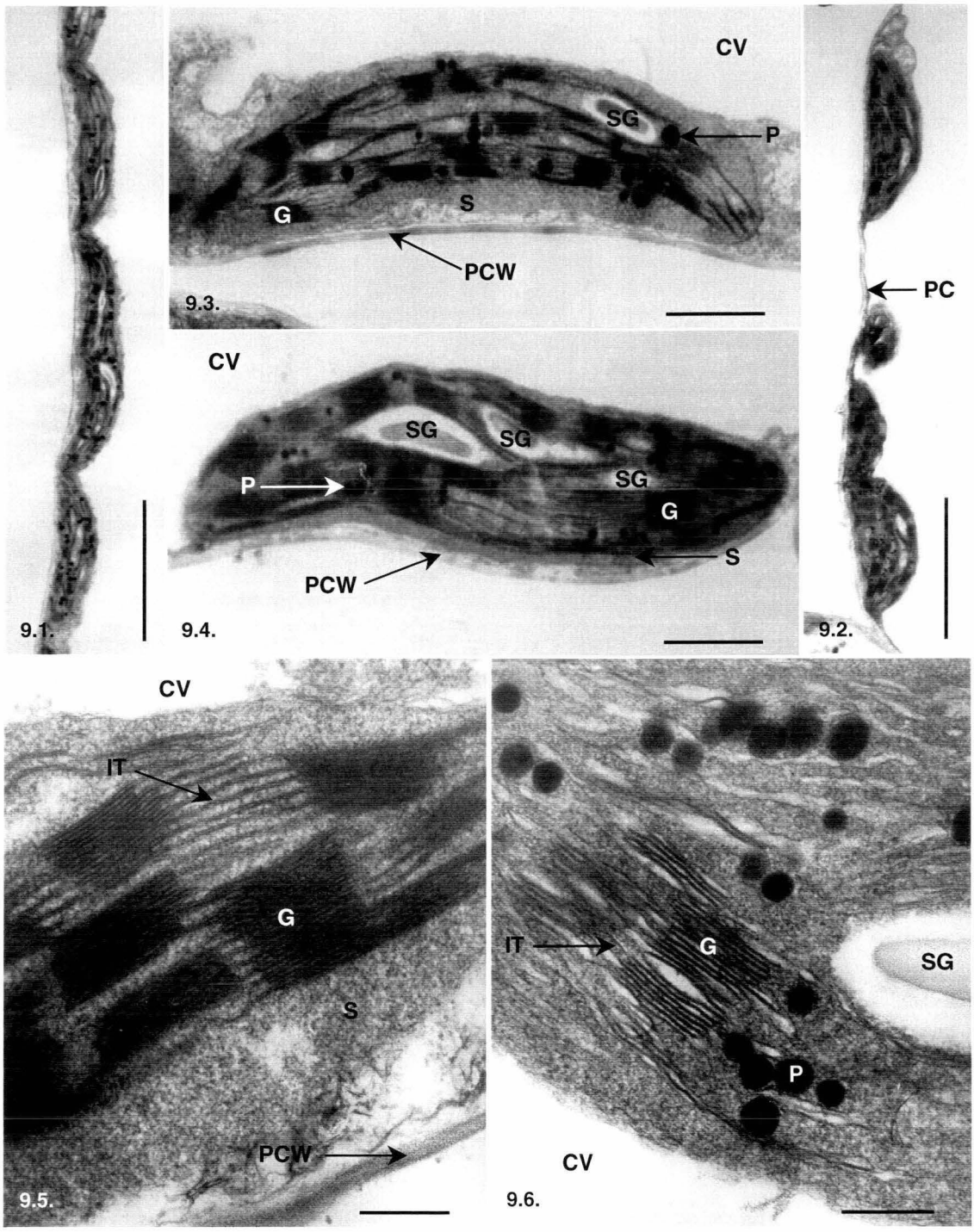

Figure 9.1. The continuous 'end-to-end' distribution of thin chloroplasts in control tissue of cultivar WL516. Bar = 5μm. **Figure 9.2.** Discrete 'bulbous' chloroplasts of NaCl-treated tissue of cultivar WL516. Note the thin peripheral cytoplasm (PC), which is devoid of chloroplasts. Bar = 5μm. **Figure 9.3.** A single thin chloroplast within control tissue of cultivar WL516. The granal stacks (G) are interspersed with dark, electron dense spherical plastoglobuli (P). Starch grains (SG) are few in number and form between granal stacks, displacing them. The plant cell wall (PCW) is discernible from the stroma (S) although the tonoplast is not distinguishable between the cytoplasm and the cell vacuole (CV). Bar = 1μm. **Figure 9.4.** A single 'bulbous' chloroplast from NaCl-treated tissue of cultivar WL516. Starch grains (SG) interspersed among grana (G), are more numerous but plastoglobuli (P) are smaller and less distinct compared to the control treatment (Fig. 3). The plant cell wall (PCW) appears to be much thicker than that in control tissue and, like control tissue, it remains distinguishable from the stroma (S). Bar = 1μm. **Figure 9.5.** In control tissue of cultivar WL516, the thylakoids within grana (G) are densely packed and intergranal thylakoids (IT) are distinct and numerous. Note the appearance of the stroma (S) and the plant cell wall (PCW). Bar = 200nm. **Figure 9.6.** A chloroplast within NaCl-treated tissue of cultivar Ameristand. Both the thylakoids comprising grana (G) and intergranal thylakoids (IT) are very loosely arranged compared to those of control tissue (Fig. 5). Note the abundant plastoglobuli (P) and large starch granule within (SG). Bar = 200nm.

CHAPTER 10

CONCLUSIONS

10. 1. INTRODUCTION

This study goes some way towards elucidating the underlying mechanisms of waterlogging and salinity tolerance in lucerne. Research into photosynthetic responses to waterlogging has shed more light on the specific response mechanisms in lucerne. Photosystem II reaction centres are damaged under waterlogged conditions, but are relatively stable under low to medium salt stress. Recovery dynamics of waterlogging were also studied and a better understanding about nutrient status under recovery conditions was gained. Photosynthetic apparatus was able to recover after waterlogging, but biomass was much reduced. Macro- and micronutrients were significantly lower in waterlogged lucerne, but recovered plants had similar nutrient concentrations to control plants. Salinity stress alters leaf anatomy and structure as well as nutrient status. It appears that lucerne genotypes employ two strategies to cope with salt stress: exclusion of Na^+ from uptake and compartmentation into vacuoles There appears to be some genotypic response variation to salt stress. Chlorophyll fluorescence studies on excised leaves exposed to salt stress revealed some genotypic responses to the parameter Fv/Fm as well as a wide response spectrum within one cultivar. This points to the possibility of a breeding strategy using tolerant individuals. Several plant physiological responses to waterlogging and salinity were assessed for their usefulness as potential screening tools for lucerne (*Medicago sativa*). The results of this study are discussed and major conclusions that can be drawn are presented below.

10. 2. CHLOROPHYLL FLUORESCENCE

The convenience and speed with which chlorophyll fluorescence readings can be obtained make it an attractive tool for use in stress physiology studies.

10. 2. 1. Waterlogging

The chlorophyll fluorescence parameter Fv/Fm appears to be a good indicator of stress severity in waterlogged lucerne under controlled glasshouse conditions. The measured values spanned a wide band of readings in stressed plants and increased with duration of the stress. However genotypic differences were difficult to detect. Lucerne is outcrossing and therefore heterozygous; this fact seemed to overshadow genotypic variability. Results of individual plants suggest that more tolerant individuals can be identified using this method. Therefore it is recommended that chlorophyll fluorescence be used in conjunction with other complementing screening tools to identify more waterlogging tolerant individuals, which can then be incorporated into further breeding programs.

10. 2. 2. Salinity

Screening excised leaves at higher salinity levels (150 mM NaCl) appeared to identify more tolerant genotypes. However, chlorophyll fluorescence did not appear to be a useful measuring tool for salinity tolerance, at least on whole plants at low and intermediate salt levels. Photosystem II is robust in response to salinity stress at least at the concentrations levels used in this study.

10. 3. PIGMENT CONCENTRATIONS

10. 3. 1. Waterlogging

Waterlogging caused a relatively rapid and progressive decline in chlorophyll *a* and *b* over time, starting 11 days after stress application. The procedure of pigment extraction is relatively time consuming and therefore not a very useful screening tool. Here the results are also overshadowed by lucerne's heterogeneity. Other instruments such as the portable chlorophyll meter SPAD 502 that measure pigment content *in situ* can possibly be employed in the search for more waterlogging tolerant genotypes and/or individuals.

10. 3. 2. Salinity

Chlorophyll concentration changes due to salinity stress appeared to take longer to manifest than in waterlogged stressed lucerne. Indeed it appeared that

chlorophyll concentrations in salt stressed lucerne increased initially due to relatively smaller leaf size and only decreased at a later stage (after three weeks) of the stress. Therefore chlorophyll concentrations do not appear to be a useful tool in early detection of salinity stress.

10. 4. NUTRIENT CONCENTRATIONS

10. 4. 1. Waterlogging

Nutrient concentration changes due to waterlogging were identified in root, stem and leaf tissues. Although there appeared to be some genotypic differences, the process of monitoring nutrient concentrations is time consuming and expensive especially when faced with the huge numbers of plants necessary for tolerance screening. As this method is destructive, individuals that might have been identified as tolerant are no longer available for breeding.

10. 4. 2. Salinity

Genotypic differences in nutrient concentrations emerged and Na^+ and Cl^- concentrations in leaves and K^+/Na^+ ratio in leaves seem to be good indicators for salinity tolerance. These indicators might also identify genotypes that use different tolerance mechanisms to cope with salinity stress. It appeared that the salt tolerant cultivar Ameristand used salt exclusion as a mechanism for tolerance whereas other genotypes possibly used sodium inclusion and sequestering into the vacuole as a way to avoid toxic stress of sodium in the cytosol.

10. 5. OTHER PHYSIOLOGICAL PARAMETERS

10. 5. 1. Waterlogging

Chlorophyll fluorescence monitoring of excised leaves in combination with exposure to salinity stress is another useful screening technique. Excised leaves of previously waterlogged plants were subjected to various levels of salinity and the results show that in this situation, where transport of toxic ions is drastically altered genotypic differences to the concomitant stress are revealed. Finetuning this technique as a screening tool may lead to a quick identification of tolerant individuals or genotypes to the dual stress of waterlogging and salinity.

10. 5. 2. Salinity

Leaf thickness correlates well with ion accumulation in leaf tissue following salt stress and might prove to be a useful preliminary indicator for varying stress responses in differing genotypes. Cell ultrastructure is altered due to salinity stress, but further studies are necessary to verify this hypothesis and to establish if genotypic responses can be identified. Since the preparation of TEM material is time consuming, TEM investigations are not suitable for screening purposes but may shed light on processes involved in tolerance mechanisms.

10. 6. MAIN CONCLUSIONS

There is sufficient variability within existing cultivars/lines of lucerne to foreshadow the improvement of salt resistance using mass selection of variable genetic material. A number of physiological parameters, affected by abiotic stresses such as waterlogging and salinity may prove useful screening tools to identify waterlogging and salinity tolerant individuals or genotypes.

Until this thesis no reports were available on how salinity and waterlogging might affect chlorophyll fluorescence characteristics in lucerne. The applicability of this screening method was therefore investigated in relation to these two environmental stresses to validate the feasibility of its use in the context of lucerne germplasm screening. Since photosystem II does not appear to be a target of salinity stress at least initially and at lower salt concentrations, screening for salinity tolerance might be better addressed by using leaf succulence characteristics together with Na^+ tissue analysis and K^+/Na^+ ratio to give an indication of different mechanisms operating regarding excess Na^+ (namely exclusion or inclusion).

Biomass by itself, although being directly linked with final yield, is not always a reliable indicator as it is influenced by a number of co-occurring stresses and environmental fluctuations. Biomass, however, is needed for validation of screening results. One of the reasons for the lack of its suitability as a screening tool is that it involves destructive harvesting and any plants identified as tolerant are eliminated from further breeding. Leaf anatomical changes such as mesophyll architecture as well as ultrastructural changes induced by stress might shed some light on mechanisms of stress responses (see chapter 9), but are time consuming to perform

and only feasible as screening tools for a small subset of genotypes selected. Finally, the non-invasive MIFE technique may prove to be a useful tool to characterize ion flux dynamics in stressed plants and provide a clear insight into subcellular functioning of membranes under stress. Although not suitable for mass screening, it can provide a better understanding e.g. of the mechanisms of ion transport processes in tolerant plants and characterize the ion flux traits in tolerant genotypes.

More detailed studies into the physiological mechanisms and strategies of lucerne exposed to waterlogging and/or salinity stress are necessary to be better equipped for breeding more tolerant crop cultivars. Also, further research is needed to verify the results and larger numbers of individuals as well as a more diverse pool of germplasm needs to be investigated for the identification of useful screening tools. Extensive field trials to assess screening results are paramount.

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